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ABSTRACTS OF THE 190TH AMERICAN CHEMICAL SOCIETY NATIONAL MEETING, vol. 190,1985, page 23, no. 47; R.R. BOTT et al.: "Protein engineering of subtilisin"

(73) Proprietor: **GENENCOR INTERNATIONAL, INC.**
180 Kimball Way
South San Francisco, CA 94080 (US)

(72) Inventor: **Wells, James Allen**
64 Otay Avenue
San Mateo
CA 94403 (US)
Inventor: **Cunningham, Brian C.**
24 Olive Avenue
Piedmont
CA 94611 (US)
Inventor: **Caldwell, Robert Mark**
1828 Broadway
No.101
San Francisco
Ca 94109 (US)
Inventor: **Bott, Richard Ray**
3032 Hillside drive
Burlingame
CA 94010 (US)

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Inventor: Estell, David Aaron
250 Diablo Avenue
Mountain View
CA 94043 (US)
Inventor: Power, Scott Douglas
732 Olive Court
San Bruno
CA 94066 (US)

(74) Representative: Armitage, Ian Michael et al
MEWBURN ELLIS
York House
23 Kingsway
London WC2B 6HP (GB)

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Description

The recent development of various in vitro techniques to manipulate the DNA sequences encoding naturally-occuring polypeptides as well as recent developments in the chemical synthesis of relatively short sequences of single and double stranded DNA has resulted in the speculation that such techniques can be used to modify enzymes to improve some functional property in a predictable way. Ulmer, K.M. (1983) Science 219, 666-671. The only working example disclosed therein is the substitution of a single amino acid within the active site of tyrosyl-tRNA synthetase (Cys35→Ser) which lead to a reduction in enzymatic activity. See Winter, G., et al. (1982) Nature 299, 756-758; and Wilkinson, A.J., et al. (1983) Biochemistry 22, 3581-3586 (Cys35→Gly mutation also resulted in decreased activity).

When the same t-RNA synthetase was modified by substituting a different amino acid residue within the active site with two different amino acids, one of the mutants (Thr51→Ala) reportedly demonstrated a predicted moderate increase in kcat/Km whereas a second mutant (Thr51→Pro) demonstrated a massive increase in kcat/Km which could not be explained with certainty. Wilkinson, A.H., et al. (1984) Nature 307, 187-188.

Another reported example of a single substitution of an amino acid residue is the substitution of cysteine for isoleucine at the third residue of T4 lysozyme. Perry, L.J., et al. (1984) Science 226, 555-557. The resultant mutant lysozyme was mildly oxidized to form a disulfide bond between the new cysteine residue at position 3 and the native cysteine at position 97. This crosslinked mutant was initially described by the author as being enzymatically identical to, but more thermally stable than, the wild type enzyme. However, in a "Note Added in Proof", the author indicated that the enhanced stability observed was probably due to a chemical modification of cysteine at residue 54 since the mutant lysozyme with a free thiol at Cys54 has a thermal stability identical to the wild type lysozyme.

Similarly, a modified dihydrofolate reductase from E.coli has been reported to be modified by similar methods to introduce a cysteine which could be cross linked with a naturally-occurring cysteine in the reductase. Villafranca, D.E., et al. (1983) Science 222, 782-788. The author indicates that this mutant is fully reactive in the reduced state but has significantly diminished activity in the oxidized state. In addition, two other substitutions of specific amino acid residues are reported which resulted in mutants which had diminished or no activity.

EPO Publication No. 0130756 discloses the substitution of specific residues within B. amyloliquefaciens subtilisin with specific amino acids. Thus, Met222 has been substituted with all 19 other amino acids, Gly166 with 9 different amino acids and Gly169 with Ala and Ser.

As set forth below, several laboratories have also reported the use of site directed mutagensis to produce the mutation of more than one amino acid residue within a polypeptide.

The amino-terminal region of the signal peptide of the prolipoprotein of the E. coli outer membrane was stated to be altered by the substitution or deletion of residues 2 and 3 to produce a charge change in that region of the polypeptide. Inoyye, S., et al. (1982) Proc. Nat. Acad. Sci. USA 79, 3438-3441. The same laboratory also reported the substitution and deletion of amino acid redisues 9 and 14 to determine the effects of such substitution on the hydrophobic region of the same signal sequence. Inouye, S., et al. (1984) J. Biol. Chem. 259, 3729-3733.

Double mutants in the active site of tyrosyl-t-RNA synthetase have also been reported. Carter, P.J., et al. (1984) Cell 38, 835-840. In this report, the improved affinity of the previously described Thr51→Pro mutant for ATP was probed by producing a second mutation in the active site of the enzyme. One of the double mutants, Gly35/Pro51, reportedly demonstrated an unexpected result in that it bound ATP in the transition state better than was expected from the two single mutants. Moreover, the author warns, at least for one double mutant, that it is not readily predictable how one substitution alters the effect caused by the other substitution and that care must be taken in interpreting such substitutions.

A mutant is disclosed in U.S. Patent No. 4,532,207, wherein a polyarginine tail was attached to the C-terminal residue of β-urogastrone by modifying the DNA sequence encoding the polypeptide. As disclosed, the polyarginine tail changed the electrophoretic mobility of the urogastrone-polyaginine hybrid permiting selective purification. The polyarginine was subsequently removed, according to the patentee, by a polyarginine specific exopeptidase to produce the purified urogastrone. Properly construed, this reference discloses hybrid polypeptides which do not constitute mutant polypeptides containing the substitution, insertion or deletion of one or more amino acids of a naturally occurring polypeptide.

Single and double mutants of rat pancreatic trypsin have also been reported. Craik, C.S., et al. (1985) Science 228, 291-297. As reported, glycine residues at positions 216 and 226 were replaced with alanine residues to produce three trypsin mutants (two single mutants and one double mutant). In the case of the single mutants, the authors stated expectation was to observe a differential effect on Km. They instead

reported a change in specificity (kcat/Km) which was primarily the result of a decrease in kcat. In contrast, the double mutant reportedly demonstrated a differential increase in Km for lysyl and arginyl substrates as compared to wild type trypsin but had virtually no catalytic activity.

The references discussed above are provided solely for their disclosure prior to the filing date of the instant case, and nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or priority based on earlier filed applications.

Based on the above references, however, it is apparent that the modification of the amino acid sequence of wild type enzymes often results in the decrease or destruction of biological activity.

Accordingly, it is an object herein to provide carbonyl hydrolase mutants which have at least one property which is different from the same property of the carbonyl hydrolase precursor from which the amino acid of said mutant is derived.

It is a further object to provide mutant DNA sequences encoding such carbonyl hydrolase mutants as well as expression vectors containing such mutant DNA sequences.

Still further, another object of the present invention is to provide host cells transformed with such vectors as well as host cells which are capable of expressing such mutants either intracellularly or extracellularly.

Summary of the Invention

The invention includes carbonyl hydrolase mutants, preferably having at least one property which is substantially different from the same property of the precursor non-human carbonyl hydrolase from which the amino acid sequence of the mutant is derived. These properties include oxidative stability, substrate, specificity catalytic activity, thermal stability, alkaline stability, pH activity profile and resistance to proteolytic degradation. The precursor carbonyl hydrolase may be naturally occurring carbonyl hydrolases or recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase mutant is derived by the substitution, deletion or insertion of one or more amino acids of the precursor carbonyl hydrolase amino acid sequence.

The invention also includes mutant DNA sequences encoding such carbonyl hydrolase mutants. Further the invention includes expression vectors containing such mutant DNA sequences as well as host cells transformed with such vectors which are capable of expressing said carbonyl hydrolase mutants.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of the coding strand, correlated with the amino acid sequence of B. amyloliquefaciens subtilisin gene. Promoter (p) ribosome binding site (rbs) and termination (term) regions of the DNA sequence as well as sequences encoding the presequence (PRE) putative prosequence (PRO) and mature form (MAT) of the hydrolase are also shown.

Figure 2 is a schematic diagram showing the substrate binding cleft of subtilisin together with substrate.

Figure 3 is a stereo view of the S-1 binding subsite of B. amyloliquefaciens subtilisin showing a lysine P-1 substrate bound in the site in two different ways. Figure 3A shows Lysine P-1 substrate bound to form a salt bridge with a Glu at position 156. Figure 3B shows Lysine P-1 substrate bound to form a salt bridge with Glu at position 166.

Figure 4 is a schematic diagram of the active site of subtilisin Asp32, His64 and Ser221.

Figures 5A and 5B depict the amino acid sequence of subtilisin obtained from various sources. The residues directly beneath each residue of B. amyloliquefaciens subtilisin are equivalent residues which (1) can be mutated in a similar manner to that described for B. amyloliquefaciens subtilisin, or (2) can be used as a replacement amino acid residue in B. amyloliquefaciens subtilisin. Figure 5C depicts conserved residues of B. amyloliquefaciens subtilisin when compared to other subtilisin sequences.

Figures 6A and 6B depict the inactivation of the mutants Met222L and Met222Q when exposed to various organic oxidants.

Figure 7 depicts the ultraviolet spectrum of Met222F subtilisin and the difference spectrum generated after inactivation by diperdodecanoic acid (DPDA).

Figure 8 shows the pattern of cyanogen bromide digests of untreated and DPDA oxidized subtilisin Met222F on high resolution SDS-pyridine peptide gels.

Figure 9 depicts a map of the cyanogen bromide fragments of Fig. 8 and their alignment with the sequence of subtilisin Met222F.

Figure 10 depicts the construction of mutations between codons 45 and 50 of B. amyloliquefaciens subtilisin.

Figure 11 depicts the construction of mutations between codons 122 and 127 of B. amyloliquefaciens subtilisin.

Figure 12 depicts the effect of DPDA on the activity of subtilisin mutants at positions 50 and 124 in subtilisin Met222F.

Figure 13 depicts the construction of mutations at codon 166 of B. amyloliquefaciens subtilisin.

Figure 14 depicts the effect of hydrophobicity of the P-1 substrate side-chain on the kinetic parameters of wild-type B. amyloliquefaciens subtilisin.

Figure 15 depicts the effect of position 166 side-chain substitutions on P-I substrate specificity. Figure 15A shows position 166 mutant subtilisins containing non-branched alkyl and aromatic side-chain substitutions arranged in order of increasing molecular volume. Figure 15B shows a series of mutant enzymes progressing through β- and γ-branched aliphatic side chain substitutions of increasing molecular volume.

Figure 16 depicts the effect of position 166 side-chain volumn on log kcat/Km for various P-1 substrates.

Figure 17 shows the substrate specificity differences between Ile166 and wild-type (Gly166) B. amyloliquefaciens subtilisin against a series of alphatic and aromatic substrates. Each bar represents the difference in log kcat/Km for Ile166 minus wild-type (Gly166) subtilisin.

Figure 18 depicts the construction of mutations at codon 169 of B. amyloliquefaciens subtilisin.

Figure 19 depicts the construction of mutations at codon 104 of B. amyloliquefaciens subtilisin.

Figure 20 depicts the construction of mutations at codon 152 B. amyloliquefaciens subtilisin.

Figure 21 depicts the construction of single mutations at codon 156 and double mutations at codons 156 and 166 of B. amyloliquefaciens subtilisin.

Figure 22 depicts the construction of mutations at codon 217 for B. amyloliquefaciens subtilisin.

Figure 23 depicts the kcat/Km versus pH profile for mutations at codon 156 and 166 in B. amyloliquefaciens subtilisin.

Figure 23A depicts the kcat/Km versus pH profile for mutations at codon 156 and 166 in B. amyloliquefaciens subtilisin.

Figure 24 depicts the kcat/Km versus pH profile for mutations at codon 222 in B. amyloliquefaciens subtilisin.

Figure 25 depicts the constructing mutants at codons 94, 95 and 96.

Figures 26 and 27 depict substrate specificity of various wild type and mutant subtilisins for different substrates.

Figures 28 A, B, C and D depict the effect of charge in the P-1 binding sites due to substitutions at codon 156 and 166.

Figures 29 A and B are a stereoview of the P-1 binding site of subtilisin BPN' showing a lysine P-1 substrate bound in the site in two ways. In 29A, Lysine P-1 substrate is built to form a salt bridge with a Glu at codon 156. In 29B, Lysine P-1 substrate is built to form a salt bridge with Glu at codon 166.

Figure 30 demonstrates residual enzyme activity versus temperature curves for purified wild-type (Panel A), C22/C87 (Panel B) and C24/C87 (Panel C).

Figure 31 depicts the strategy for producing point mutations in the subtilisin coding sequence by misincorporation of $^{\alpha}$-thioldeoxynucleotide triphosphates.

Figure 32 depicts the autolytic stability of purified wild type and mutant subtilisins 170E, 107V, 213R and 107V/213R at alkaline pH.

Figure 33 depicts the autolytic stability of purified wild type and mutant subtilisins V50, F50 and F50/V107/R213 at alkaline pH.

Figure 34 depicts the strategy for constructing plasmids containing random cassette mutagenesis over residues 197 through 228.

Figure 35 depicts the oligodeoxynucleotides used for random cassette mutagenesis over residues 197 through 228.

Figure 36 depicts the construction of mutants at codon 204.

Figure 37 depicts the oligodeoxynucleotides used for synthesizing mutants at codon 204.

Detailed Description

The inventors have discovered that various single and multiple in vitro mutations involving the substitution, deletion or insertion of one or more amino acids within a non-human carbonyl hydrolase amino acid sequence can confer advantageous properties to such mutants when compared to the non-mutated carbonyl hydrolase.

Specifically, *B. amyloliquefaciens* subtilisin, an alkaline bacterial protease, has been mutated by modifying the DNA encoding the subtilisin to encode the substitution of one or more amino acids at various amino acid residues within the mature form of the subtilisin molecule. These *in vitro* mutant subtilisins have at least one property which is different when compared to the same property of the precursor subtilisin. These modified properties fall into several categories including: oxidative stability, substrate specificity, thermal stability, alkaline stability, catalytic activity, pH activity profile, resistance to proteolytic degradation, Km, kcat and Km/kcat ratio.

Carbonyl hydrolases are enzymes which hydrolyze compounds containing

$$\begin{array}{c} O \\ \| \\ C{-}X \end{array}$$

bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally occurring carbonyl hydrolases principally include hydrolases, e.g. lipases and peptide hydrolases, e.g. subtilisins or metalloproteases. Peptide hydrolases include α-aminoacylpeptide hydrolase, peptidylamino-acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exoproteases.

"Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes the substitution, insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein and in EPO Publication No. 0130756 published January 9, 1985.

Subtilisins are bacterial carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally occurring subtilisin or a recombinant subtilisin. A series of naturally occurring subtilisins is known to be produced and often secreted by various bacterial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus is aspartate-histidineserine. In the chymotrypsin related proteases the relative order, however is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases.

"Recombinant subtilisin" refers to a subtilisin in which the DNA sequence encoding the subtilisin is modified to produce a mutant DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally occurring subtilisin amino acid sequence. Suitable methods to produce such modification include those disclosed herein and in EPO Publication No. 0130756. For example, the subtilisin multiple mutant herein containing the substitution of methionine at amino acid residues 50, 124 and 222 with phenylalanine, isoleucine and glutamine, respectively, can be considered to be derived from the recombinant subtilisin containing the substitution of glutamine at residue 222 (Q222) disclosed in EPO Publication No. 0130756. The multiple mutant thus is produced by the substitution of phenylalanine for methionine at residue 50 and isoleucine for methionine at residue 124 in the Q222 recombinant subtilisin.

"Carbonyl hydrolases" and their genes may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or pseudomonas and gram positive bacteria such as micrococcus or bacillus. Examples of eucaryotic organisms from which carbonyl hydrolase and their genes may be obtained include yeast such as *S. cerevisiae*, fungi such as Aspergillus sp., and non-human mammalian sources such as, for example, Bovine sp. from which the gene encoding the carbonyl hydrolase chymosin can be obtained. As with subtilisins, a series of carbonyl hydrolases can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a functional definition which refers to carbonyl hydrolases which are associated, directly or indirectly, with procaryotic and non-human eucaryotic sources.

A "carbonyl hydrolase mutant" has an amino acid sequence which is derived from the amino acid sequence of a non-human "precursor carbonyl hydrolase". The precursor carbonyl hydrolases include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase mutant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase rathern than manipulation of the precursor carbonyl hydrolase per se. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein and in EPO Publication No. 0130756.

Specific residues of B. amyloliquefaciens subtilisin are identified for substitution, insertion or deletion. These amino acid position numbers refer to those assigned to the B. amyloliquefaciens subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor carbonyl hydrolases containing amino acid residues which are "equivalent" to the particular identified residues in B. amyloliquefaciens subtilisin.

A residue (amino acid) of a precursor carbonyl hydrolase is equivalent to a residue of B. amyloliquefaciens subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analagous to a specific residue or portion of that residue in B. amyloliquefaciens subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly comparted to the B. amyloliquefaciens subtilisin primary sequence and particularly to a set of residues known to be invariant in all subtilisins for which sequence is known (Figure 5C). After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of B. amyloliquefaciens subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, in Figure 5A the amino acid sequence of subtilisin from B. amyloliquefaciens B. subtilisin var. I168 and B. lichenformis (carlsbergensis) are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These residues are identified in Fig. 5C.

These conserved residues thus may be used to define the corresponding equivalent amino acid residues of B. amyloliquefaciens subtilisin in other carbonyl hydrolases such as thermitase derived from Thermoactinomyces. These two particular sequences are aligned in Fig. 5B to produce the maximum homology of conserved residues. As can be seen there are a number of insertions and deletions in the thermitase sequence as compared to B. amyloliquefaciens subtilisin. Thus, in thermitase the equivalent amino acid of Tyr217 in B. amyloliquefaciens subtilisin is the particular lysine shown beneath Tyr217.

In Fig. 5A, the equivalent amino acid at position 217 in B. amyloliquefaciens subtilisin is Tyr. Likewise, in B. subtilis subtilisin position 217 is also occupied by Tyr but in B. licheniformis position 217 is occupied by Leu.

Thus, these particular residues in thermitase, and subtilisin from B. subtilisin and B. licheniformis may be substituted by a different amino acid to produce a mutant carbonyl hydrolase since they are equivalent in primary structure to Tyr217 in B. amyloliquefaciens subtilisin. Equivalent amino acids of course are not limited to those for Tyr217 but extend to any residue which is equivalent to a residue in B. amyloliquefaciens whether such residues are conserved or not.

Equivalent residues homologous at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography, are defined as those for which the atomic coordinates of 2 or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and B. amyloliquefaciens subtilisin (N on N, CA on CA, C on C, and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the B. amyloliquefaciens subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$\text{R factor} = \frac{\sum_h |Fo(h)| - |Fc(h)|}{\sum_h |Fo(h)|}$$

Equivalent residues which are functionally analogous to a specific residue of B. amyloliquefaciens subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the B. amyloliquefaciens subtilisin as described herein. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography), which occupy an analogous position to the extent that although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of B. amyloliquefaciens subtilisin. The three dimensional structures would be aligned as outlined above.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a mutant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally occurring sequence. The carbonyl hydrolase mutants of the present invention include the mature forms of carbonyl hydrolase mutants as well as the pro- and prepro-forms of such hydrolase mutants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase mutants.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in EPO Publication No. 0130756 to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing subtilisin is the Bacillus strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in EPO Publicatin No. 0130756 and further described by Yang, M.Y., et al. (1984) J. Bacteriol. 160, 15-21. Other host cells for expressing subtilisin include Bacillus subtilis I168 (EPO Publication No. 0130756).

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the carbonyl hydrolase mutants or expressing the desired carbonyl hydrolase mutant. In the case of vectors which encode the pre or prepro form of the carbonyl hydrolase mutant, such mutants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the general methods described herein in EPO publication No. 0130756.

Once the carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant carbonyl hydrolases as disclosed in EPO

Publication No. 0130756 and the production of carbonyl hydrolase mutants described herein.

The carbonyl hydrolase mutants of the present invention may be generated by site specific mutagenesis (Smith, M. (1985) Ann, Rev. Genet. 423; Zoeller, M.J., et al. (1982) Nucleic Acid Res. 10, 6487-6500), cassette mutagenesis (EPO Publication No. 0130756) or random mutagenesis (Shortle, D., et al. (1985) Genetics, 110, 539; Shortle, D., et al. (1986) Proteins: Structure, Function and Genetics, 1, 81; Shortle, D. (1986) J. Cell. Biochem, 30, 281; Alber, T., et al. (1985) Proc. Natl. Acad. of Sci., 82, 747; Matsumura, M., et al. (1985) J. Biochem., 260, 15298; Liao, H., et al. (1986) Proc. Natl. Acad. of Sci., 83 576) of the cloned precursor carbonyl hydrolase. Cassette mutagenesis and the random mutagenesis method disclosed herein are preferred.

The mutant carbonyl hydrolases expressed upon transformation of suitable hosts are screened for enzymes exhibiting one or more properties which are substantially different from the properties of the precursor carbonyl hydrolases, e.g., changes in substrate specificity, oxidative stability, thermal stability, alkaline stability, resistance to proteolytic degradation, pH-activity profiles and the like.

A change in substrate specificity is defined as a difference between the kcat/Km ratio of the precursor carbonyl hydrolase and that of the hydrolase mutant. The kcat/Km ratio is a measure of catalytic efficienty. Carbonyl hydrolase mutants with increased or diminished kcat/Km ratios are described in the examples. Generally, the objective will be to secure a mutant having a greater (numerically large) kcat/Km ratio for a given substrate, thereby enabling the use of the enzyme to more efficiently act on a target substrate. A substantial change in kcat/Km ratio is preferably at least 2-fold increase or decrease. However, smaller increases or decreases in the ratio (e.g., at least 1.5-fold) are also considered substantial. An increase in kcat/Km ratio for one substrate may be accompanied by a reduction in kcat/Km ratio for another substrate. This is a shift in substrate specificity, and mutants exhibiting such shifts have utility where the precursor hydrolase is undesirable, e.g. to prevent undesired hydrolysis of a particular substrate in an admixture of substrates. Km and kcat are measured in accord with known procedures, as described in EPO Publication No. 0130756 or as described herein.

Oxidative stability is measured either by known procedures or by the methods described hereinafter. A substantial change in oxidative stability is evidenced by at least about 50% increase or decrease (preferably decrease) in the rate of loss of enzyme activity when exposed to various oxidizing conditions. Such oxidizing conditions are exposure to the organic oxidant diperdodecanoic acid (DPDA) under the conditions described in the examples.

Alkaline stability is measured either by known procedures or by the methods described herein. A substantial change in alkaline stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half life of the enzymatic activity of a mutant when compared to the precursor carbonyl hydrolase. In the case of subtilisins, alkaline stability was measured as a function of autoproteolytic degradation of subtilisin at alkaline pH, e.g. for example, 0.1M sodium phosphate, pH 12 at 25° or 30°C.

Thermal stability is measured either by known procedures or by the methods described herein. A substantial change in thermal stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half-life of the catalytic activity of a mutant when exposed to a relatively high temperature and neutral pH as compared to the precursor carbonyl hydrolase. In the case of subtilisins, thermal stability is measured by the autoproteolytic degradation of subtilisin at elevated temperatures and neutral pH, e.g., for example 2mM calcium chloride, 50mM MOPS pH 7.0 at 59°C.

The inventors have produced mutant subtilisins containing the substitution of the amino acid residues of B. amyloliquefaciens subtilisin shown in Table I. The wild type amino acid sequence and DNA sequence of B. amyloliquefaciens subtilisin is shown in Fig. 1.

TABLE I

Residue	Replacement Amino Acid
Tyr21	F A
Thr22	C
Ser24	C
Asp32	Q S
Ser33	A T
Asp36	A G
Gly46	V
Ala48	E V R
Ser49	C L
Met50	C F V
Asn77	D
Ser87	C
Lys94	C
Val95	C
Leu96	D
Tyr104	A C D E F G H I K L M N P Q R S T V W
Ile107	V
Gly110	C R
Met124	I L
Asn155	A D H Q T
Glu156	Q S
Gly166	C E I L M P S T W Y
Gly169	C D E F H I K L M N P Q R T V W Y
Lys170	E R
Tyr171	F
Pro172	E Q
Phe189	A C D E G H I K L M N P Q R S T V W Y
Asp197	R A
Met199	I
Ser204	C R L P
Lys213	R T
Tyr217	A C D E F G H I K L M N P Q R S T V W
Ser221	A C

The different amino acids substituted are represented in Table I by the following single letter designations:

Amino acid or residue thereof	3-letter symbol	1-letter symbol
Alanine	Ala	A
Glutamate	Glu	E
Glutamine	Gln	Q
Aspartate	Asp	D
Asparagine	Asn	N
Leucine	Leu	L
Glycine	Gly	G
Lysine	Lys	K
Serine	Ser	S
Valine	Val	V
Arginine	Arg	R
Threonine	Thr	T
Proline	Pro	P
Isoleucine	Ile	I
Methionine	Met	M
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Cysteine	Cys	C
Tryptophan	Trp	W
Histidine	His	H

Except where otherwise indicated by context, wild-type amino acids are represented by the above three-letter symbols and replaced amino acids by the above single-letter symbols. Thus, if the methionine at residue 50 in B. amyloliquefaciens subtilisin is replaced by phenylalanine, this mutation (mutant) may be designated Met50F or F50. Similar designations are used for multiple mutants.

In addition to the amino acids used to replace the residues disclosed in Table I, other replacements of amino acids at these residues are expected to produce mutant subtilisins having useful properties. These residues and replacement amino acids are shown in Table II.

TABLE II

Residue	Replacement Amino Acid(s)
Tyr-21	L
Thr22	K
Ser24	A
Asp32	
Ser33	G
Gly46	
Ala48	
Ser49	
Met50	L K I V
Asn77	D
Ser87	N
Lys94	R Q
Val95	L I
Tyr104	
Met124	K A
Ala152	C L I T M
Asn155	
Glu156	A T M L Y
Gly166	
Gly169	
Tyr171	K R E Q
Pro172	D N
Phe189	
Tyr217	
Ser221	
Met222	

Each of the mutant subtilisins in Table I contain the replacement of a single residue of the B. amyloliquefaciens amino acid sequence. These particular residues were chosen to probe the influence of such substitutions on various properties of B. amyloliquefacien subtilisin.

Thus, the inventors have identified Met124 and Met222 as important residues which if substituted with another amino acid produce a mutant subtilisin with enhanced oxidative stability. For Met124, Leu and Ile are preferred replacement amino acids. Preferred amino acids for replacement of Met222 are disclosed in EPO Publication No. 0130756.

Various other specific residues have also been identified as being important with regard to substrate specificity. These residues include Tyr104, Ala152, Glu156, Gly166, Gly169, Phe189 and Tyr217 for which mutants containing the various replacement amino acids presented in Table I have already been made, as well as other residues presented below for which mutants have yet to be made.

The identification of these residues, including those yet to be mutated, is based on the inventors' high resolution crystal structure of B. amyloliquefaciens subtilisin to 1.8 A (see Table III), their experience with in vitro mutagenesis of subtilisin and the literature on subtilisin. This work and the x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449), product complexes (Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303), and transition state analogs (Matthews, D.A., et al (1975) J. Biol. Chem. 250, 7120-7126; Poulos, T.L., et al. (1976) J. Biol. Chem. 251, 1097-1103), has helped in identifying an extended peptide binding cleft in subtilisin. This substrate binding cleft together with substrate is schematically diagramemed in Fig. 2, according to the nomenclature of Schechter, I., et al. (1967) Biochem Bio. Res. Commun. 27, 157. The scissile bond in the substrate is identified by an arrow. The P and P' designations refer to the amino acids which are positioned respectively toward the amino or carboxy terminus relative to the scissle bond. The S and S' designations refer to subsites in the substrate binding cleft of subtilisin which interact with the corresponding substrate amino acid residues.

Atomic Coordinates for the Apoenzyme Form of B, Amyloliquefaciens Subtilisin to 1.8AResolution

1	ALA N	19.436	53.195	-21.756	1	ALA CA	19.811	51.774	-21.965
1	ALA C	18.731	50.925	-21.324	1	ALA O	18.376	51.197	-20.175
1	ALA CB	21.099	51.518	-21.183	2	GLN N	18.268	49.886	-22.041
2	GLN CA	17.219	49.008	-21.434	2	GLN C	17.875	47.786	-20.992
2	GLN O	18.765	47.165	-21.691	2	GLN CB	16.125	48.760	-22.449
2	GLN CG	15.328	47.905	-21.927	2	GLN CD	13.912	47.762	-22.930
2	GLN OE1	13.023	48.612	-22.867	2	GLN NE2	14.115	46.917	-23.926
3	SER N	17.477	47.205	-19.852	3	SER CA	17.950	45.868	-19.437
3	SER C	16.735	44.918	-19.490	3	SER O	15.590	45.352	-19.229
3	SER CB	18.588	45.038	-18.069	3	SER OG	17.682	46.218	-17.049
4	VAL N	16.991	43.646	-19.725	4	VAL CA	15.946	42.619	-19.639
4	VAL C	16.129	41.934	-18.290	4	VAL O	17.123	41.178	-18.886
4	VAL CB	16.008	43.622	-20.822	4	VAL CG1	14.876	40.572	-20.761
4	VAL CG2	16.037	42.266	-22.186	5	PRO N	15.239	42.186	-17.331
5	PRO CA	15.384	41.415	-18.027	5	PRO C	15.501	39.905	-16.249
5	PRO O	14.885	39.263	-17.146	5	PRO CB	14.150	41.880	-15.263
5	PRO CG	13.841	43.215	-15.921	5	PRO CD	14.844	42.986	-17.417
6	TYR N	16.363	39.240	-15.487	6	TYR CA	16.628	37.883	-15.715
6	TYR C	15.359	36.975	-15.528	6	TYR O	15.224	35.943	-16.235
6	TYR CB	17.824	37.323	-14.834	6	TYR CG	18.021	35.847	-15.855
6	TYR CD1	18.437	35.452	-16.346	6	TYR CD2	17.696	34.988	-14.871
6	TYR CE1	18.535	34.070	-16.653	6	TYR CE2	17.815	33.539	-14.379
6	TYR CZ	18.222	33.154	-15.628	6	TYR OH	18.312	31.838	-15.996
7	GLY N	14.464	37.362	-14.630	7	GLY CA	13.211	36.640	-14.376
7	GLY C	12.400	36.535	-15.670	7	GLY O	11.747	35.678	-15.883
8	VAL N	12.461	37.529	-16.541	8	VAL CA	11.777	37.523	-17.836
8	VAL C	12.363	36.433	-18.735	8	VAL O	11.639	35.716	-19.470
8	VAL CB	11.765	38.900	-18.567	8	VAL CG1	11.186	38.893	-19.943
8	VAL CG2	10.991	39.919	-17.733	9	SER N	13.661	36.318	-18.775
9	SER CA	14.419	35.342	-19.562	9	SER C	14.188	33.920	-18.965
9	SER O	14.112	33.014	-19.801	9	SER CB	15.926	35.632	-19.505
9	SER OG	16.162	36.747	-20.358	10	GLN N	14.115	33.887	-17.662
10	GLN CA	13.964	32.636	-16.876	10	GLN C	12.687	31.887	-17.277
10	GLN O	12.785	30.642	-17.413	10	GLN CB	14.125	32.885	-15.418
10	GLN CG	14.295	31.617	-14.588	10	GLN CD	14.486	31.911	-13.147
10	GLN OE1	14.554	33.068	-12.746	10	GLN NE2	14.552	30.960	-12.251
11	ILE N	11.625	32.575	-17.670	11	ILE CA	10.373	31.904	-18.182
11	ILE C	10.209	31.792	-19.605	11	ILE O	9.173	31.333	-20.180
11	ILE CB	9.132	32.669	-17.475	11	ILE CG1	9.066	34.117	-18.049
11	ILE CG2	9.162	32.655	-15.941	11	ILE CD1	7.588	34.648	-17.923
12	LYS N	11.272	32.185	-20.277	12	LYS CA	11.388	32.119	-21.722
12	LYS C	10.456	33.006	-22.522	12	LYS O	10.178	32.703	-23.686
12	LYS CB	11.257	30.646	-22.216	12	LYS CG	12.283	29.830	-21.423
12	LYS CD	12.543	28.517	-22.159	12	LYS CE	13.023	27.467	-21.166
12	LYS NZ	14.476	27.680	-20.935	13	ALA N	10.109	34.138	-21.991
13	ALA CA	9.325	35.198	-22.631	13	ALA C	10.026	35.716	-23.863
13	ALA O	9.338	35.804	-24.901	13	ALA CB	8.885	36.195	-21.565
14	PRO N	11.332	35.950	-23.893	14	PRO CA	11.985	36.438	-25.128
14	PRO C	11.786	35.557	-26.317	14	PRO O	11.778	36.047	-27.445
14	PRO CB	13.462	36.588	-24.692	14	PRO CG	13.328	36.978	-23.221
14	PRO CD	12.281	35.936	-22.758	15	ALA N	11.560	34.236	-26.129
15	ALA CA	11.379	33.458	-27.367	15	ALA C	10.082	33.795	-28.032
15	ALA O	10.008	33.718	-29.278	15	ALA CB	11.552	31.969	-27.062
16	LEU N	9.085	34.138	-27.248	16	LEU CA	7.791	34.558	-27.828
16	LEU C	7.912	35.925	-28.521	16	LEU O	7.342	36.126	-29.588
16	LEU CB	6.746	34.623	-26.698	16	LEU CG	5.798	33.465	-26.522
16	LEU CD1	5.881	33.234	-27.889	16	LEU CD2	6.694	32.287	-26.283
17	HIS N	8.665	36.828	-27.922	17	HIS CA	8.898	38.151	-28.538
17	HIS C	9.518	37.981	-29.898	17	HIS O	9.187	38.622	-30.856
17	HIS CB	9.708	39.188	-27.652	17	HIS CG	9.185	39.288	-26.262
17	HIS ND1	9.938	39.887	-25.272	17	HIS CD2	8.088	38.926	-25.694
17	HIS CE1	9.226	39.914	-24.144	17	HIS NE2	8.079	39.328	-26.381
18	SER N	10.443	37.833	-30.822	18	SER CA	11.189	36.739	-31.322

18	SER	C	10.159	36.123	-32.393
18	SER	CB	12.311	35.799	-31.172
19	GLN	N	9.880	35.695	-31.963
19	GLN	C	7.142	36.111	-33.303
19	GLN	CB	7.221	33.869	-32.280
19	GLN	CD	6.923	31.707	-31.181
19	GLN	NE2	7.362	30.852	-30.256
20	GLY	CA	6.369	38.387	-32.859
20	GLY	O	4.263	39.276	-32.215
21	TYR	CA	4.118	37.831	-29.763
21	TYR	O	5.422	38.876	-27.756
21	TYR	CG	2.973	35.784	-30.798
21	TYR	CD2	3.650	36.796	-31.397
21	TYR	CE2	3.193	34.261	-32.881
21	TYR	OH	1.301	34.241	-34.250
22	THR	CA	6.262	40.327	-27.129
22	THR	O	3.287	41.725	-25.325
22	THR	OG1	6.319	42.457	-28.597
23	GLY	N	1.939	40.285	-26.453
23	GLY	C	-0.257	41.631	-26.118
24	SER	N	-0.823	41.967	-27.371
24	SER	C	-2.383	42.626	-27.864
24	SER	CB	-0.734	43.128	-29.520
25	ASN	N	-3.059	43.692	-27.515
25	ASN	C	-5.018	42.873	-26.203
25	ASN	CB	-5.165	43.227	-28.703
25	ASN	OD1	-4.965	43.767	-31.083
26	VAL	N	-4.377	42.449	-25.292
26	VAL	C	-6.792	42.652	-22.997
26	VAL	CB	-3.714	40.903	-23.821
26	VAL	CG2	-3.598	39.576	-25.018
27	LYS	CA	-6.133	43.526	-21.173
27	LYS	O	-6.405	41.873	-19.413
27	LYS	CG	-8.046	44.575	-22.490
27	LYS	CE	-10.304	45.497	-23.137
28	VAL	N	-4.818	43.462	-18.203
28	VAL	C	-4.758	43.959	-16.828
28	VAL	CB	-2.926	42.666	-17.932
28	VAL	CG2	-2.667	41.805	-19.173
29	ALA	CA	-5.747	44.330	-14.639
29	ALA	O	-6.666	42.845	-13.104
30	VAL	N	-6.857	45.033	-13.072
30	VAL	C	-3.958	45.609	-10.681
30	VAL	CB	-1.886	45.810	-12.149
30	VAL	CG2	-1.853	45.236	-13.307
31	ILE	CA	-5.328	44.866	-8.679
31	ILE	O	-3.828	43.915	-6.997
31	ILE	CG1	-7.298	43.707	-9.798
31	ILE	CD1	-8.617	42.856	-9.717
32	ASP	CA	-2.966	46.467	-6.255
32	ASP	O	-4.397	48.418	-8.302
32	ASP	CG	-0.483	45.702	-6.273
32	ASP	OD2	-0.881	46.628	-5.330
33	SER	CA	-1.895	49.857	-4.801
33	SER	O	-1.706	52.136	-5.363
33	SER	OG	0.535	50.025	-4.776
34	GLY	CA	-2.855	51.728	-8.165
34	GLY	O	-5.166	50.931	-8.761
35	ILE	CA	0.208	52.638	-10.995
35	ILE	O	-0.327	54.638	-11.766
35	ILE	CG1	-0.530	50.210	-12.097
35	ILE	CD1	-0.962	49.485	-13.426
36	ASP	CA	2.359	55.618	-11.232
18	SER	O	10.947	36.332	-33.836
18	SER	OG	13.323	36.450	-30.399
19	GLN	CA	8.882	36.962	-32.878
19	GLN	O	6.297	35.972	-34.219
19	GLN	CG	7.978	32.602	-31.823
19	GLN	OE1	5.719	31.033	-31.666
20	GLY	N	7.288	37.223	-32.887
20	GLY	C	5.181	38.692	-31.888
21	TYR	N	5.282	37.801	-30.761
21	TYR	C	4.879	38.552	-28.825
21	TYR	CB	3.698	36.631	-29.663
21	TYR	CD1	2.795	36.332	-31.288
21	TYR	CE1	3.306	35.797	-32.666
21	TYR	CZ	2.803	34.755	-33.967
22	THR	N	5.902	39.680	-28.288
22	THR	C	5.091	40.822	-26.344
22	THR	CB	5.133	41.759	-27.611
22	THR	CG2	6.476	43.323	-28.229
23	GLY	CA	0.809	40.600	-25.562
23	GLY	O	-1.833	42.895	-25.330
24	SER	CA	-0.897	42.957	-28.012
24	SER	O	-2.833	41.508	-28.160
24	SER	OG	0.563	43.632	-29.720
25	ASN	CA	-4.519	43.687	-27.393
25	ASN	O	-6.233	42.668	-26.190
25	ASN	CG	-4.960	44.170	-29.885
25	ASN	ND2	-4.747	45.461	-29.896
26	VAL	CA	-4.674	41.679	-24.143
26	VAL	O	-3.858	43.439	-22.689
26	VAL	CG1	-4.160	39.802	-22.948
27	LYS	N	-5.930	42.613	-22.301
27	LYS	C	-5.815	42.872	-19.841
27	LYS	CB	-7.890	43.981	-21.169
27	LYS	CD	-9.321	45.302	-22.820
27	LYS	NZ	-9.686	46.253	-24.264
28	VAL	CA	-4.637	42.980	-17.097
28	VAL	O	-4.289	45.095	-16.017
28	VAL	CG1	-2.486	42.103	-16.589
29	ALA	N	-5.484	43.527	-15.813
29	ALA	C	-6.750	44.010	-13.353
29	ALA	CB	-7.172	44.187	-14.181
30	VAL	CA	-3.146	44.982	-11.910
30	VAL	O	-4.155	46.648	-10.878
30	VAL	CG1	-0.906	45.901	-10.980
31	ILE	N	-4.516	44.515	-9.077
31	ILE	C	-4.346	44.933	-7.846
31	ILE	CB	-6.457	43.776	-8.901
31	ILE	CG2	-7.278	44.038	-7.225
32	ASP	N	-4.844	46.193	-7.227
32	ASP	C	-3.871	47.889	-5.703
32	ASP	CB	-1.695	46.129	-7.092
32	ASP	OD1	0.834	44.392	-6.576
33	SER	N	-1.931	48.512	-5.396
33	SER	C	-1.982	50.976	-5.888
33	SER	CB	-0.621	49.922	-3.939
34	GLY	N	-2.173	50.760	-7.086
34	GLY	C	-1.035	51.668	-9.057
35	ILE	N	-0.965	52.431	-10.102
35	ILE	C	0.568	53.939	-11.263
35	ILE	CB	-0.062	53.696	-12.367
35	ILE	CG2	1.169	53.741	-13.362
36	ASP	N	1.016	54.253	-10.971
36	ASP	C	2.281	55.956	-12.702

36	ASP	O	3.004	55.471	-13.579
36	ASP	CG	4.339	57.099	-10.806
36	ASP	OD2	5.440	57.277	-10.263
37	SER	CA	1.103	57.221	-14.512
37	SER	O	2.545	58.303	-16.151
37	SER	OG	-0.090	59.133	-13.079
38	SER	CA	4.261	59.505	-14.407
38	SER	O	6.543	59.251	-15.285
38	SER	OG	5.376	59.865	-12.234
39	HIS	CA	6.637	56.574	-15.291
39	HIS	O	5.738	55.078	-17.419
39	HIS	CG	8.014	56.609	-14.456
39	HIS	CD2	8.769	54.345	-13.389
39	HIS	NE2	9.986	53.910	-13.008
40	PRO	CA	7.988	56.697	-18.831
40	PRO	O	8.832	55.097	-20.370
40	PRO	CG	10.053	57.405	-17.902
41	ASP	N	8.461	54.328	-18.485
41	ASP	OD1	10.325	51.395	-20.429
41	ASP	CB	9.799	52.239	-18.226
41	ASP	C	7.311	52.163	-18.839
42	LEU	N	6.185	52.803	-18.558
42	LEU	C	3.924	52.907	-19.376
42	LEU	CB	4.421	52.158	-17.008
42	LEU	CD1	4.535	51.546	-16.581
43	LYS	N	3.018	52.135	-19.946
43	LYS	C	0.637	52.156	-20.018
43	LYS	CB	2.021	52.389	-22.169
43	LYS	CD	0.998	52.862	-24.339
43	LYS	NZ	0.337	51.757	-26.418
44	VAL	CA	-1.407	52.639	-18.765
44	VAL	O	-2.623	53.906	-20.434
44	VAL	CG1	-2.724	52.941	-16.582
45	ALA	N	-3.494	51.951	-19.071
45	ALA	C	-5.841	52.507	-20.053
45	ALA	CB	-4.031	50.580	-21.389
46	GLY	CA	-7.082	52.037	-18.001
46	GLY	O	-5.938	52.006	-16.035
47	GLY	CA	-8.014	52.246	-14.388
47	GLY	O	-9.988	53.481	-14.185
48	ALA	CA	-10.235	52.070	-11.382
48	ALA	O	-9.066	51.720	-9.725
49	SER	N	-10.149	53.547	-9.037
49	SER	C	-10.947	52.986	-6.783
49	SER	CB	-9.092	54.588	-7.029
50	MET	N	-10.835	52.087	-5.932
50	MET	C	-11.463	51.962	-3.561
50	MET	CB	-12.812	50.818	-4.996
50	MET	SD	-13.460	49.819	-7.256
51	VAL	N	-10.427	52.760	-3.422
51	VAL	C	-10.630	54.562	-1.987
51	VAL	CB	-8.443	53.135	-2.808
51	VAL	CG2	-7.764	53.815	-2.302
52	PRO	CA	-12.372	55.933	-0.821
52	PRO	O	-11.771	58.220	-0.925
52	PRO	CG	-13.583	54.103	0.085
53	SER	N	-10.442	56.986	0.299
53	SER	C	-8.428	58.245	-0.326
53	SER	CB	-9.004	57.707	2.069
54	GLU	N	-8.254	57.523	-1.393
54	GLU	C	-7.767	57.303	-3.785
54	GLU	CB	-6.134	56.599	-2.156
54	GLU	CD	-6.044	56.067	-0.970

36	ASP	CB	3.712	55.720	-10.516
36	ASP	OD1	3.755	57.974	-11.429
37	SER	N	1.304	56.022	-13.111
37	SER	C	2.377	58.095	-14.949
37	SER	CB	-0.093	58.049	-14.788
38	SER	N	3.163	58.614	-14.081
38	SER	C	5.466	58.705	-14.992
38	SER	CB	4.762	60.435	-13.398
39	HIS	N	5.454	57.398	-14.892
39	HIS	C	6.681	56.401	-16.778
39	HIS	CB	6.637	55.203	-14.515
39	HIS	ND1	8.795	54.356	-15.561
39	HIS	CE1	9.970	53.938	-15.130
40	PRO	N	7.007	56.836	-17.387
40	PRO	C	8.156	55.280	-19.357
40	PRO	CB	9.247	57.533	-19.161
40	PRO	CD	8.988	57.452	-16.776
41	ASP	OD2	11.140	50.399	-18.668
41	ASP	CG	10.473	51.387	-19.211
41	ASP	CA	8.445	52.959	-18.966
41	ASP	O	7.396	50.947	-18.977
42	LEU	CA	4.892	52.147	-18.466
42	LEU	O	3.993	54.163	-19.490
42	LEU	CG	5.182	51.363	-15.946
42	LEU	CD2	5.273	49.877	-16.358
43	LYS	CA	2.893	52.605	-20.721
43	LYS	O	0.504	50.920	-19.820
43	LYS	CG	0.605	52.436	-22.910
43	LYS	CE	-0.100	52.504	-25.260
44	VAL	N	-0.191	53.035	-19.490
44	VAL	C	-2.571	52.887	-19.731
44	VAL	CB	-1.480	53.351	-17.383
44	VAL	CG2	-0.197	53.194	-16.553
45	ALA	CA	-4.619	51.977	-20.810
45	ALA	O	-6.783	53.085	-20.783
46	GLY	N	-5.918	52.356	-18.768
46	GLY	C	-6.987	52.443	-16.538
47	GLY	N	-8.092	52.658	-15.793
47	GLY	C	-9.179	52.757	-13.572
48	ALA	N	-9.221	52.446	-12.330
48	ALA	C	-9.790	52.675	-9.968
48	ALA	CB	-11.558	52.100	-11.617
49	SER	CA	-9.752	53.355	-7.652
49	SER	O	-11.972	53.677	-6.908
49	SER	OG	-8.379	54.255	-5.650
50	MET	CA	-11.852	51.549	-4.974
50	MET	O	-11.997	51.398	-2.575
50	MET	CG	-11.912	49.463	-6.389
50	MET	CE	-12.808	50.111	-8.983
51	VAL	CA	-9.968	53.178	-2.067
51	VAL	O	-10.237	55.437	-2.682
51	VAL	CG1	-7.892	53.579	-0.631
52	PRO	N	-11.621	54.693	-1.056
52	PRO	C	-11.490	57.123	-0.440
52	PRO	CB	-13.400	55.594	0.244
52	PRO	CD	-12.264	53.620	-0.175
53	SER	CA	-9.538	57.982	0.602
53	SER	O	-7.679	59.224	-0.038
53	SER	OG	-8.256	56.521	2.127
54	GLU	CA	-7.204	57.660	-2.421
54	GLU	O	-7.533	56.243	-4.379
54	GLU	CG	-5.209	56.959	-0.927
54	GLU	OE1	-5.565	55.606	-1.968

54	GLU OE2	-3.908	55.777	0.271	55	THR N	-8.571	58.251	-4.249
55	THR CA	-9.433	58.121	-5.441	55	THR C	-9.764	58.139	-6.779
55	THR O	-9.433	57.919	-7.810	55	THR CB	-10.586	59.200	-5.303
55	THR OG1	-9.885	60.510	-5.410	55	THR CG2	-11.432	59.143	-4.817
56	ASN N	-7.482	58.403	-6.877	56	ASN ND2	-6.930	61.179	-9.881
56	ASN OD1	-5.875	58.967	-10.337	56	ASN CG	-5.273	59.925	-9.555
56	ASN CB	-5.898	59.494	-8.208	56	ASN CA	-6.762	58.425	-8.280
56	ASN C	-6.812	57.994	-8.305	56	ASN O	-5.184	56.866	-7.678
57	PRO N	-6.342	56.261	-9.258	57	PRO CG	-7.123	55.257	-11.177
57	PRO CD	-7.304	56.433	-10.272	57	PRO CB	-6.644	54.178	-10.235
57	PRO CA	-5.679	54.961	-9.332	57	PRO C	-4.301	55.882	-9.966
57	PRO O	-3.509	54.128	-9.965	58	PHE N	-3.988	56.262	-10.491
58	PHE CA	-2.747	56.577	-11.222	58	PHE C	-1.712	57.129	-10.253
58	PHE O	-0.635	57.497	-10.680	58	PHE CB	-2.943	57.502	-12.623
58	PHE CG	-3.983	56.968	-13.357	58	PHE CD1	-3.756	55.700	-14.859
58	PHE CD2	-5.211	57.630	-13.459	58	PHE CE1	-4.722	55.255	-14.928
58	PHE CE2	-6.194	57.095	-14.276	58	PHE CZ	-5.949	55.939	-15.851
59	GLN N	-2.044	57.119	-8.990	59	GLN CA	-1.172	57.583	-7.934
59	GLN C	-0.807	56.403	-7.800	59	GLN O	-1.639	56.883	-6.115
59	GLN CB	-1.862	58.668	-7.889	59	GLN CG	-0.942	59.261	-6.834
59	GLN CD	-1.790	60.157	-5.150	59	GLN OE1	-1.404	61.288	-6.836
59	GLN NE2	-2.959	59.485	-4.762	60	ASP N	0.410	55.895	-7.211
60	ASP CA	0.851	54.792	-6.304	60	ASP C	1.631	55.267	-5.090
60	ASP O	2.827	55.550	-5.231	60	ASP CB	1.596	53.744	-7.188
60	ASP CG	2.077	52.530	-6.380	60	ASP OD1	1.746	52.337	-5.190
60	ASP OD2	2.915	51.861	-7.830	61	ASN N	0.959	55.265	-3.950
61	ASN ND2	-1.364	57.747	-2.347	61	ASN OD1	0.666	58.566	-2.875
61	ASN CG	-0.040	57.670	-2.399	61	ASN CB	0.531	56.401	-1.784
61	ASN CA	1.557	55.734	-2.700	61	ASN C	2.291	54.632	-1.940
61	ASN O	2.933	54.862	-0.902	62	ASN N	2.210	53.434	-2.468
62	ASN CA	2.877	52.348	-1.709	62	ASN C	4.124	51.893	-2.479
62	ASN O	4.951	51.313	-1.770	62	ASN CB	1.783	51.319	-1.621
62	ASN CG	2.371	50.103	-0.697	62	ASN OD1	2.633	49.877	-1.343
62	ASN ND2	2.622	50.208	0.601	63	SER N	4.152	52.184	-3.761
63	SER CA	5.189	51.696	-4.709	63	SER C	5.071	50.256	-5.209
63	SER O	5.593	49.790	-6.269	63	SER CB	6.523	51.958	-6.812
63	SER OG	6.871	50.698	-3.418	64	HIS N	4.202	49.475	-4.639
64	HIS CA	3.994	48.855	-4.935	64	HIS C	3.366	47.759	-6.261
64	HIS O	3.861	46.974	-7.108	64	HIS CB	3.184	47.501	-3.747
64	HIS CG	3.144	46.021	-3.726	64	HIS ND1	2.107	45.247	-4.241
64	HIS CD2	4.056	45.194	-3.135	64	HIS CE1	2.416	43.966	-4.054
64	HIS NE2	3.556	43.920	-3.368	65	GLY N	2.287	48.420	-6.587
65	GLY CA	1.552	48.264	-7.830	65	GLY C	2.392	48.636	-9.037
65	GLY O	2.230	48.078	-10.134	66	THR N	3.233	49.659	-8.832
66	THR CA	4.064	50.117	-9.954	66	THR C	5.889	49.809	-10.291
66	THR O	5.333	48.789	-11.461	66	THR CB	4.744	51.511	-9.667
66	THR OG1	3.637	52.425	-9.406	66	THR CG2	5.536	52.870	-10.849
67	HIS N	5.685	48.443	-9.274	67	HIS CA	6.703	47.361	-9.458
67	HIS C	6.091	46.141	-10.143	67	HIS O	6.649	45.638	-11.150
67	HIS CB	7.300	47.871	-8.064	67	HIS CG	8.595	46.275	-8.148
67	HIS ND1	8.590	46.907	-8.276	67	HIS CD2	9.904	46.678	-8.076
67	HIS CE1	9.857	44.491	-8.299	67	HIS NE2	10.678	45.514	-8.186
68	VAL N	4.892	45.749	-9.731	68	VAL CA	4.142	44.687	-10.266
68	VAL C	3.856	44.860	-11.740	68	VAL O	4.114	43.942	-12.535
68	VAL CB	2.939	44.252	-9.386	68	VAL CG1	1.960	43.260	-10.820
68	VAL CG2	3.319	43.705	-8.888	69	ALA N	3.373	46.049	-12.113
69	ALA CA	3.837	46.468	-13.429	69	ALA C	4.193	46.390	-14.411
69	ALA O	4.828	45.913	-15.565	69	ALA CB	2.332	47.851	-13.386
70	GLY N	5.348	46.782	-13.914	70	GLY CA	6.595	46.805	-14.670
70	GLY C	7.046	45.378	-15.821	70	GLY O	7.684	45.154	-16.119
71	THR N	6.820	44.631	-14.138	71	THR CA	7.177	43.019	-14.446
71	THR C	6.224	42.506	-15.543	71	THR O	6.682	41.828	-16.495
71	THR CB	7.119	42.870	-13.191	71	THR OG1	8.191	42.592	-12.390

71	THR	CG2	7.274	40.503	-13.596	72	VAL	N	4.930	42.007	-15.427
72	VAL	CA	3.976	42.491	-16.484	72	VAL	C	4.312	43.004	-17.031
72	VAL	O	4.341	42.300	-18.860	72	VAL	CB	2.916	42.867	-16.085
72	VAL	CG1	1.512	42.490	-17.170	72	VAL	CG2	2.142	42.327	-14.723
73	ALA	N	4.534	44.417	-17.880	73	ALA	CA	4.587	45.091	-19.167
73	ALA	C	5.433	46.333	-19.355	73	ALA	O	5.062	47.188	-20.216
73	ALA	CB	3.107	45.441	-19.433	74	ALA	N	6.566	46.429	-18.635
74	ALA	CA	7.470	47.591	-18.959	74	ALA	C	7.740	47.648	-20.342
74	ALA	O	7.959	46.640	-21.054	74	ALA	CB	8.653	47.446	-17.925
75	LEU	N	7.650	48.784	-21.839	75	LEU	CA	7.812	48.968	-22.456
75	LEU	C	9.192	48.568	-22.966	75	LEU	O	10.162	48.758	-22.253
75	LEU	CB	7.548	50.471	-22.809	75	LEU	CG	6.123	50.913	-22.379
75	LEU	CD1	6.079	52.436	-22.300	75	LEU	CD2	5.096	50.442	-23.405
76	ASN	N	9.147	48.103	-24.169	76	ASN	ND2	12.385	46.432	-26.304
76	ASN	OD1	10.950	45.840	-27.928	76	ASN	CG	11.195	46.274	-26.082
76	ASN	CB	10.010	46.651	-25.908	76	ASN	CA	10.359	47.738	-24.938
76	ASN	C	10.783	49.048	-25.643	76	ASN	O	10.157	49.479	-26.619
77	ASN	N	11.804	49.664	-25.071	77	ASN	CA	12.220	50.957	-25.601
77	ASN	C	13.707	51.029	-25.348	77	ASN	O	14.364	49.979	-25.333
77	ASN	CB	11.335	52.876	-25.117	77	ASN	CG	11.250	52.027	-23.616
77	ASN	OD1	12.032	51.346	-22.917	77	ASN	ND2	10.294	52.741	-23.825
78	SER	N	14.125	52.267	-25.164	78	SER	CA	15.513	52.614	-24.906
78	SER	C	15.810	52.742	-23.436	78	SER	O	16.982	53.071	-23.164
78	SER	CB	15.905	53.943	-25.587	78	SER	OG	15.926	53.070	-26.999
79	ILE	N	14.858	52.565	-22.529	79	ILE	CA	15.155	52.784	-21.120
79	ILE	C	14.617	51.683	-20.230	79	ILE	O	13.843	50.841	-20.679
79	ILE	CB	14.471	54.174	-20.697	79	ILE	CG1	12.945	54.832	-20.814
79	ILE	CG2	14.997	55.320	-21.612	79	ILE	CD1	12.135	55.176	-20.155
80	GLY	N	14.995	51.768	-18.981	80	GLY	CA	14.476	50.940	-17.913
80	GLY	C	14.612	49.448	-18.219	80	GLY	O	15.719	48.994	-18.546
81	VAL	N	13.513	48.766	-17.980	81	VAL	CA	13.421	47.286	-18.061
81	VAL	C	12.511	46.919	-19.217	81	VAL	O	12.260	47.739	-20.117
81	VAL	CB	13.001	46.755	-16.677	81	VAL	CG1	14.030	47.084	-15.573
81	VAL	CG2	11.638	47.261	-16.231	82	LEU	N	12.126	45.645	-19.216
82	LEU	CA	11.312	45.020	-20.256	82	LEU	C	10.390	44.028	-19.510
82	LEU	O	10.858	43.356	-18.600	82	LEU	CB	12.286	44.219	-21.229
82	LEU	CG	11.430	43.568	-22.366	82	LEU	CD1	10.796	44.657	-23.223
82	LEU	CD2	12.359	42.675	-23.192	83	GLY	N	9.131	44.180	-19.816
83	GLY	CA	8.133	43.321	-19.114	83	GLY	C	8.027	42.811	-19.925
83	GLY	O	8.546	41.822	-21.026	84	VAL	N	7.272	41.112	-19.283
84	VAL	CA	6.973	39.807	-19.888	84	VAL	C	6.164	40.030	-21.140
84	VAL	O	6.424	39.472	-22.194	84	VAL	CB	6.256	38.920	-18.841
84	VAL	CG1	5.680	37.677	-19.557	84	VAL	CG2	7.190	38.507	-17.705
85	ALA	N	5.156	40.926	-21.024	85	ALA	CA	4.217	41.194	-22.158
85	ALA	C	4.213	42.683	-22.396	85	ALA	O	3.260	43.401	-22.030
85	ALA	CB	2.846	40.663	-21.748	86	PRO	N	5.240	43.186	-23.859
86	PRO	CA	5.413	44.635	-23.285	86	PRO	C	4.321	45.371	-23.947
86	PRO	O	4.291	46.605	-23.849	86	PRO	CB	6.822	44.784	-23.813
86	PRO	CG	7.030	43.466	-24.546	86	PRO	CD	6.377	42.440	-23.636
87	SER	N	3.548	44.676	-24.769	87	SER	CA	2.489	45.324	-25.529
87	SER	C	1.303	45.132	-24.897	87	SER	O	0.162	45.513	-25.619
87	SER	CB	2.401	44.777	-26.927	87	SER	OG	3.591	45.143	-27.583
88	ALA	N	1.017	44.564	-23.742	88	ALA	CB	-0.163	43.510	-21.828
88	ALA	CA	-0.273	44.353	-23.084	88	ALA	C	-0.899	45.717	-22.690
88	ALA	O	-0.174	46.717	-22.435	89	SER	N	-2.219	45.691	-22.678
89	SER	OG	-4.146	47.102	-24.280	89	SER	CB	-4.343	46.903	-22.898
89	SER	CA	-3.001	46.867	-22.227	89	SER	C	-3.136	46.780	-20.727
89	SER	O	-3.793	45.864	-20.209	90	LEU	N	-2.446	47.656	-20.037
90	LEU	CA	-2.370	47.667	-18.593	90	LEU	C	-3.483	48.430	-17.864
90	LEU	O	-3.582	49.604	-18.215	90	LEU	CB	-0.951	48.273	-18.476
90	LEU	CG	-0.233	47.851	-17.174	90	LEU	CD1	-0.026	46.341	-17.219
90	LEU	CD2	1.160	49.524	-17.047	91	TYR	N	-4.264	47.964	-16.938
91	TYR	CA	-5.258	48.678	-16.137	91	TYR	C	-4.873	48.758	-14.685

91	TYR	O	-6.496	47.749	-16.023
91	TYR	CG	-7.094	48.237	-17.741
91	TYR	CD2	-7.971	49.275	-18.149
91	TYR	CE2	-8.315	49.421	-19.492
91	TYR	OH	-8.102	48.752	-21.764
92	ALA	CA	-4.949	50.199	-12.707
92	ALA	O	-6.723	50.898	-12.050
93	VAL	N	-5.959	48.993	-11.129
93	VAL	C	-4.708	49.814	-8.899
93	VAL	CB	-7.957	47.555	-10.631
93	VAL	CG2	-8.195	47.378	-12.072
94	LYS	CA	-6.378	50.464	-6.999
94	LYS	O	-8.458	50.400	-5.783
94	LYS	CG	-5.394	52.320	-5.467
94	LYS	CE	-4.399	54.208	-4.199
95	VAL	N	-6.909	49.071	-5.026
95	VAL	C	-6.919	48.499	-2.568
95	VAL	CB	-8.106	47.030	-4.319
95	VAL	CG2	-6.900	46.100	-4.332
96	LEU	CA	-4.782	49.103	-1.486
96	LEU	O	-3.942	51.121	-2.336
96	LEU	CG	-3.593	46.799	-2.072
96	LEU	CD2	-4.489	46.082	-1.845
97	GLY	CA	-3.890	52.307	0.287
97	GLY	O	-1.619	51.463	0.165
98	ALA	CB	-0.420	55.478	1.510
98	ALA	C	0.188	53.118	1.917
99	ASP	N	-0.504	52.573	2.912
99	ASP	OD1	-2.730	50.902	4.003
99	ASP	CB	-0.648	51.603	5.175
99	ASP	C	0.146	50.165	3.320
100	GLY	N	-0.424	49.083	2.168
100	GLY	C	-1.520	47.651	2.002
101	SER	N	-2.342	48.128	2.908
101	SER	C	-4.759	47.894	2.532
101	SER	CB	-3.716	47.447	4.817
102	GLY	N	-5.021	47.892	2.577
102	GLY	C	-8.166	46.536	2.520
103	GLN	N	-9.377	47.058	2.490
103	GLN	C	-10.963	45.232	2.022
103	GLN	CB	-11.671	47.307	3.274
103	GLN	CD	-12.368	49.106	4.915
103	GLN	NE2	-13.419	49.197	4.112
104	TYR	CA	-12.068	43.126	1.508
104	TYR	O	-12.939	43.276	-0.087
104	TYR	CG	-11.629	40.029	2.472
104	TYR	CD2	-10.379	40.959	1.860
104	TYR	CE2	-9.352	40.057	2.371
104	TYR	OH	-8.481	38.191	3.326
105	SER	CA	-14.077	45.166	-0.036
105	SER	O	-14.759	45.935	-2.258
105	SER	OG	-15.209	47.039	1.450
106	TRP	CA	-12.421	47.391	-1.948
106	TRP	O	-12.021	46.648	-4.245
106	TRP	CG	-11.645	49.111	-0.206
106	TRP	CD2	-10.658	49.812	0.981
106	TRP	CE2	-11.359	50.573	1.561
106	TRP	CZ2	-10.671	51.310	2.500
106	TRP	CH2	-9.293	51.291	2.455
107	ILE	CA	-10.765	44.250	-3.325
107	ILE	O	-11.695	43.474	-5.398
107	ILE	CG1	-8.636	43.784	-1.936
107	ILE	CD1	-8.283	42.998	-0.627
91	TYR	CB	-6.686	48.093	-16.314
91	TYR	CD1	-6.595	47.415	-18.755
91	TYR	CE1	-6.985	47.572	-20.098
91	TYR	CZ	-7.794	48.582	-20.463
92	ALA	N	-4.895	49.958	-14.104
92	ALA	C	-5.823	50.833	-11.903
92	ALA	CB	-3.997	51.621	-12.488
93	VAL	CA	-7.183	48.854	-10.325
93	VAL	O	-6.181	47.993	-8.372
93	VAL	CG1	-9.213	47.488	-9.725
94	LYS	N	-6.907	50.217	-8.327
94	LYS	C	-7.331	49.985	-5.894
94	LYS	CB	-6.051	51.976	-6.818
94	LYS	CD	-4.068	53.785	-5.582
94	LYS	NZ	-3.735	55.944	-4.387
95	VAL	CA	-7.646	48.457	-3.920
95	VAL	O	-7.425	48.156	-1.581
95	VAL	CG1	-8.868	46.852	-5.619
96	LEU	N	-5.676	48.974	-2.604
96	LEU	C	-6.331	50.559	-1.321
96	LEU	CB	-3.509	48.241	-1.573
96	LEU	CD1	-2.207	46.184	-2.163
97	GLY	N	-4.326	50.975	-0.086
97	GLY	C	-2.363	52.437	0.385
98	ALA	N	-1.954	53.648	0.758
98	ALA	CA	-0.563	54.068	0.965
98	ALA	O	1.393	52.921	1.663
99	ASP	OD2	-2.631	51.042	6.151
99	ASP	CG	-2.083	51.131	5.048
99	ASP	CA	0.181	51.618	3.855
99	ASP	O	0.735	49.313	4.029
100	GLY	CA	-0.363	48.521	1.615
100	GLY	O	-1.649	46.512	1.479
101	SER	CA	-3.542	47.388	3.315
101	SER	O	-4.758	48.972	1.907
101	SER	OG	-4.411	48.634	5.209
102	GLY	CA	-7.077	47.422	1.896
102	GLY	O	-7.088	45.431	3.030
103	GLN	CA	-10.535	46.297	3.020
103	GLN		-10.779	45.482	0.817
103	GLN	CG	-11.368	48.005	4.586
103	GLN	OE1	-12.159	49.816	5.902
104	TYR	N	-11.611	44.143	2.451
104	TYR	C	-13.031	43.690	0.473
104	TYR	CB	-12.697	41.866	2.143
104	TYR	CD1	-11.819	39.789	3.377
104	TYR	CE1	-10.809	38.885	3.707
104	TYR	CZ	-9.564	39.022	3.081
105	SER	N	-13.909	44.572	0.983
105	SER	C	-14.172	45.920	-1.159
105	SER	CB	-15.000	46.121	0.601
106	TRP	N	-13.079	46.625	-0.834
106	TRP	C	-11.895	46.436	-3.012
106	TRP	CB	-11.321	48.254	-1.355
106	TRP	CD1	-12.862	49.524	0.264
106	TRP	NE1	-12.691	50.358	1.360
106	TRP	CE3	-9.275	49.852	0.576
106	TRP	CZ3	-8.568	50.563	1.525
107	ILE	N	-11.339	45.330	-2.481
107	ILE	C	-11.955	43.994	-4.190
107	ILE	CB	-9.944	43.183	-2.923
107	ILE	CG2	-9.632	41.930	-3.381
108	ILE	N	-12.994	43.292	-3.577

108	ILE	CA	-14.116	42.722	-4.321	108	ILE	C	-14.639	43.694	-5.386
108	ILE	O	-14.894	43.329	-6.552	108	ILE	CB	-15.266	42.265	-3.320
108	ILE	CG1	-14.726	41.077	-2.482	108	ILE	CG2	-16.568	42.824	-4.895
108	ILE	CD1	-15.452	40.845	-1.331	109	ASN	N	-14.751	44.958	-6.981
109	ASN	CA	-15.204	46.018	-5.916	109	ASN	C	-14.232	46.067	-7.886
109	ASN	O	-14.660	46.272	-8.235	109	ASN	CB	-15.288	47.359	-5.287
109	ASN	CG	-16.528	47.486	-6.353	109	ASN	OD1	-17.695	46.695	-6.646
109	ASN	ND2	-16.633	48.447	-3.442	110	GLY	N	-12.951	45.908	-6.774
110	GLY	CA	-11.952	45.917	-7.865	110	GLY	C	-12.188	44.712	-8.812
110	GLY	O	-11.929	44.929	-10.834	111	ILE	N	-12.379	43.539	-8.246
111	ILE	CA	-12.603	42.334	-9.099	111	ILE	C	-13.859	42.560	-9.942
111	ILE	O	-13.921	42.384	-11.140	111	ILE	CB	-12.734	40.948	-8.366
111	ILE	CG1	-11.421	40.501	-7.655	111	ILE	CG2	-13.122	39.791	-9.347
111	ILE	CD1	-11.588	39.786	-6.336	112	GLU	N	-14.893	43.875	-9.280
112	GLU	CA	-16.118	43.376	-10.846	112	GLU	C	-15.872	44.347	-11.171
112	GLU	O	-16.467	44.130	-12.246	112	GLU	CB	-17.229	43.899	-9.141
112	GLU	CG	-17.847	42.917	-8.135	112	GLU	CD	-18.724	41.824	-8.685
112	GLU	OE1	-19.841	40.866	-8.816	112	GLU	OE2	-19.123	41.928	-9.866
113	TRP	N	-15.894	45.403	-10.971	113	TRP	CA	-14.756	46.408	-12.888
113	TRP	C	-14.876	45.663	-13.140	113	TRP	O	-14.319	45.932	-14.332
113	TRP	CB	-13.882	47.553	-11.434	113	TRP	CG	-13.486	48.556	-12.481
113	TRP	CD1	-14.148	49.736	-12.681	113	TRP	CD2	-12.441	48.552	-13.463
113	TRP	NE1	-13.597	50.443	-13.723	113	TRP	CE2	-12.545	49.761	-14.215
113	TRP	CE3	-11.451	47.645	-13.809	113	TRP	CZ2	-11.696	50.845	-15.274
113	TRP	CZ3	-10.610	47.899	-14.879	113	TRP	CH2	-10.752	49.874	-15.603
114	ALA	N	-13.089	44.801	-12.832	114	ALA	CA	-12.333	44.865	-13.874
114	ALA	C	-13.199	43.179	-14.752	114	ALA	O	-12.963	43.874	-15.978
114	ALA	CB	-11.299	43.192	-13.140	115	ILE	N	-14.174	42.540	-16.119
115	ILE	CA	-15.070	41.640	-14.897	115	ILE	C	-15.928	42.485	-15.856
115	ILE	O	-16.077	42.225	-17.070	115	ILE	CB	-16.000	40.840	-13.922
115	ILE	CG1	-15.218	39.836	-13.843	115	ILE	CG2	-17.151	40.168	-14.755
115	ILE	CD1	-16.004	39.411	-11.743	116	ALA	N	-16.534	43.527	-15.267
116	ALA	CA	-17.390	44.440	-16.850	116	ALA	C	-16.786	45.869	-17.278
116	ALA	O	-17.323	45.255	-18.343	116	ALA	CB	-18.811	45.510	-15.151
117	ASN	N	-15.423	45.390	-17.122	117	ASN	CA	-16.553	45.967	-18.139
117	ASN	C	-13.827	44.974	-19.834	117	ASN	O	-12.997	45.636	-19.820
117	ASN	CB	-13.615	46.958	-17.426	117	ASN	CG	-14.400	48.177	-16.939
117	ASN	OD1	-14.565	49.882	-17.773	117	ASN	ND2	-14.931	48.249	-15.736
118	ASN	N	-14.223	43.725	-18.967	118	ASN	CA	-13.760	42.662	-19.832
118	ASN	C	-12.240	42.444	-19.843	118	ASN	O	-11.617	42.309	-20.932
118	ASN	CB	-14.247	42.863	-21.279	118	ASN	CG	-15.737	43.060	-21.395
118	ASN	OD1	-16.510	42.321	-20.759	118	ASN	ND2	-16.136	44.096	-22.133
119	MET	N	-11.686	42.500	-18.675	119	MET	CA	-10.232	42.222	-18.478
119	MET	C	-10.825	40.734	-18.928	119	MET	O	-10.888	39.830	-18.759
119	MET	CB	-9.810	42.461	-17.055	119	MET	CG	-9.880	43.883	-16.582
119	MET	SD	-8.788	44.943	-17.526	119	MET	CE	-9.982	46.061	-18.263
120	ASP	N	-8.904	40.637	-19.584	120	ASP	CA	-8.488	39.118	-20.030
120	ASP	C	-7.822	38.390	-18.856	120	ASP	O	-8.038	37.189	-18.690
120	ASP	CB	-7.555	39.156	-21.236	120	ASP	CG	-8.237	39.730	-22.454
120	ASP	OD1	-7.881	40.706	-23.084	120	ASP	OD2	-9.327	39.135	-22.739
121	VAL	N	-7.021	39.117	-18.115	121	VAL	CA	-6.226	38.601	-16.974
121	VAL	C	-6.296	39.534	-15.786	121	VAL	O	-6.284	40.788	-15.909
121	VAL	CB	-4.755	38.507	-17.496	121	VAL	CG1	-3.758	38.176	-16.427
121	VAL	CG2	-4.787	37.916	-18.046	122	ILE	N	-6.318	38.978	-14.590
122	ILE	CA	-6.248	39.799	-13.397	122	ILE	C	-5.828	39.262	-12.627
122	ILE	O	-4.829	38.012	-12.469	122	ILE	CB	-7.476	39.604	-12.466
122	ILE	CG1	-8.686	40.392	-13.063	122	ILE	CG2	-7.221	39.883	-10.954
122	ILE	CD1	-9.976	39.788	-12.393	123	ASN	N	-4.263	40.222	-12.118
123	ASN	CA	-3.145	39.856	-11.232	123	ASN	C	-3.582	40.404	-9.863
123	ASN	O	-3.788	41.631	-9.833	123	ASN	CB	-1.828	40.478	-11.697
123	ASN	CG	-0.692	40.848	-10.777	123	ASN	OD1	-0.863	38.990	-11.818
123	ASN	ND2	-0.346	40.747	-9.720	124	MET	N	-3.458	39.604	-8.832
124	MET	CA	-3.650	39.973	-7.638	124	MET	C	-2.423	39.603	-6.614

124	MET	O	-2.306	38.908	-6.893	124	MET	CB	-6.963	38.387	-6.890
124	MET	CG	-6.198	40.082	-7.673	124	MET	SD	-7.985	39.672	-6.450
124	MET	CE	-7.969	38.095	-7.942	125	SER	N	-1.696	40.696	-6.902
125	SER	CA	-0.193	40.287	-5.769	125	SER	C	-0.622	40.712	-6.326
125	SER	O	0.235	41.617	-3.005	125	SER	CB	1.021	41.027	-6.328
125	SER	OG	1.666	40.496	-7.575	126	LEU	N	-1.633	40.095	-3.775
126	LEU	CA	-1.842	40.367	-2.306	126	LEU	C	-2.438	39.856	-1.807
126	LEU	O	-2.866	38.136	-2.529	126	LEU	CB	-2.781	41.960	-2.610
126	LEU	CG	-3.988	41.667	-3.333	126	LEU	CD1	-5.278	41.331	-2.578
126	LEU	CD2	-6.179	42.760	-4.073	127	GLY	N	-2.922	39.082	-0.681
127	GLY	CA	-3.035	37.871	0.193	127	GLY	C	-3.176	38.180	1.602
127	GLY	O	-2.666	39.030	2.220	128	GLY	N	-6.121	37.443	2.222
128	GLY	CA	-6.675	37.496	3.642	128	GLY	C	-6.666	36.038	4.106
128	GLY	O	-6.983	35.158	3.276	129	PRO	N	-6.519	35.897	5.602
129	PRO	CA	-6.671	36.325	5.998	129	PRO	C	-6.116	34.886	6.082
129	PRO	O	-6.338	32.887	6.305	129	PRO	CB	-6.060	36.686	7.386
129	PRO	CG	-6.619	36.116	7.727	129	PRO	CD	-6.239	36.870	6.618
130	SER	N	-7.051	35.015	5.912	130	SER	CA	-8.670	36.611	6.023
130	SER	C	-9.218	36.886	4.726	130	SER	O	-8.969	35.881	6.829
130	SER	CB	-9.069	35.351	7.216	130	SER	OG	-8.723	36.626	8.603
131	GLY	N	-10.003	33.967	4.369	131	GLY	CA	-10.826	36.229	3.076
131	GLY	C	-12.205	36.713	3.862	131	GLY	O	-12.695	36.722	4.781
132	SER	N	-13.960	33.038	2.896	132	SER	CA	-16.607	35.633	3.011
132	SER	C	-15.289	36.805	1.936	132	SER	O	-16.799	36.816	0.026
132	SER	CB	-16.590	36.927	3.145	132	SER	OG	-16.693	37.339	1.075
133	ALA	N	-16.967	36.588	2.206	133	ALA	CA	-17.507	36.057	1.326
133	ALA	C	-17.630	36.965	0.097	133	ALA	O	-17.763	36.437	-1.036
133	ALA	CB	-18.866	33.020	1.996	134	ALA	N	-17.683	36.288	0.296
134	ALA	CA	-17.072	37.239	-0.792	134	ALA	C	-16.635	37.369	-1.676
134	ALA	O	-16.781	37.585	-2.869	134	ALA	CB	-18.263	38.600	-0.107
135	LEU	N	-15.478	37.229	-1.066	135	LEU	CA	-16.197	37.266	-1.806
135	LEU	C	-16.198	36.005	-2.765	135	LEU	O	-13.796	36.020	-3.890
135	LEU	CB	-13.038	37.328	-0.798	135	LEU	CG	-11.693	37.130	-1.501
135	LEU	CD1	-11.460	38.635	-2.292	135	LEU	CD2	-10.582	36.007	-0.519
136	LYS	N	-16.909	36.825	-2.173	136	LYS	CA	-16.563	33.597	-3.013
136	LYS	C	-15.566	33.739	-6.150	136	LYS	C	-15.279	33.631	-5.305
136	LYS	CB	-16.903	32.361	-2.186	136	LYS	CG	-16.763	31.067	-3.063
136	LYS	CD	-15.003	29.892	-2.136	136	LYS	CE	-15.763	28.707	-2.778
136	LYS	NZ	-15.300	28.611	-6.160	137	ALA	N	-16.766	36.260	-3.067
137	ALA	CA	-17.795	36.616	-6.083	137	ALA	C	-17.338	35.303	-6.065
137	ALA	O	-17.705	35.069	-7.208	137	ALA	CB	-19.096	36.961	-6.263
138	ALA	N	-16.329	36.301	-5.729	138	ALA	CA	-16.001	37.311	-6.605
138	ALA	C	-16.903	36.696	-7.557	138	ALA	O	-16.985	36.863	-8.762
138	ALA	CB	-15.522	38.567	-5.936	139	VAL	N	-13.950	35.959	-7.027
139	VAL	CA	-12.966	35.291	-7.837	139	VAL	C	-13.623	36.228	-8.720
139	VAL	O	-13.208	36.070	-9.877	139	VAL	CB	-11.830	36.671	-6.968
139	VAL	CG1	-10.919	33.836	-7.866	139	VAL	CG2	-11.078	35.780	-6.233
140	ASP	N	-16.893	33.336	-8.122	140	ASP	CA	-15.276	32.696	-8.929
140	ASP	C	-16.023	33.131	-10.006	140	ASP	O	-16.080	32.579	-11.190
140	ASP	CB	-16.169	31.569	-8.135	140	ASP	CG	-15.388	30.640	-7.186
140	ASP	OD1	-16.170	30.603	-7.282	140	ASP	OD2	-16.139	30.132	-6.329
141	LYS	N	-16.653	34.263	-9.020	141	LYS	CA	-17.373	35.006	-10.868
141	LYS	C	-18.373	35.618	-11.946	141	LYS	O	-16.700	35.268	-13.111
141	LYS	CB	-18.039	36.275	-10.325	141	LYS	CG	-18.886	37.836	-11.306
141	LYS	CD	-19.606	38.187	-10.536	141	LYS	CE	-20.572	39.051	-11.230
141	LYS	NZ	-21.138	40.037	-10.275	142	ALA	N	-15.167	35.840	-11.566
142	ALA	CA	-16.173	36.192	-12.616	142	ALA	C	-13.818	35.010	-13.321
142	ALA	O	-13.770	35.169	-14.755	142	ALA	CB	-12.870	36.697	-11.968
143	VAL	N	-13.582	33.886	-12.832	143	VAL	CA	-13.168	32.785	-13.650
143	VAL	C	-16.346	32.233	-16.496	143	VAL	O	-16.160	31.886	-15.639
143	VAL	CB	-12.531	31.673	-12.716	143	VAL	CG1	-12.300	30.370	-13.661
143	VAL	CG2	-11.303	32.195	-12.016	144	ALA	N	-15.531	32.238	-13.875
144	ALA	CA	-16.766	31.836	-16.061	144	ALA	C	-16.928	32.881	-15.861

144	ALA	C	-17.380	32.263	-16.958	144	ALA	CB	-17.942	31.968	-13.788
145	SER	N	-16.507	33.948	-15.706	145	SER	CA	-16.682	34.937	-16.786
145	SER	C	-15.609	34.773	-17.829	145	SER	O	-15.918	35.321	-18.893
145	SER	CB	-17.016	36.376	-16.616	145	SER	OG	-15.882	36.935	-15.849
146	GLY	N	-14.577	33.086	-17.565	146	GLY	CA	-13.619	33.799	-18.675
146	GLY	C	-12.273	34.491	-18.385	146	GLY	O	-11.620	34.386	-19.266
147	VAL	N	-12.150	35.162	-17.254	147	VAL	CA	-10.876	35.856	-16.912
147	VAL	C	-9.850	34.836	-16.323	147	VAL	O	-10.171	33.991	-15.686
147	VAL	CB	-11.152	36.977	-15.089	147	VAL	CG1	-9.896	37.803	-15.578
147	VAL	CG2	-12.360	37.913	-16.230	148	VAL	N	-8.083	35.818	-16.683
148	VAL	CA	-7.482	34.230	-16.008	148	VAL	C	-7.157	34.907	-14.701
148	VAL	O	-6.865	36.133	-14.750	148	VAL	CB	-6.273	34.126	-16.958
148	VAL	CG1	-5.079	33.483	-16.281	148	VAL	CG2	-6.598	33.632	-18.262
149	VAL	N	-7.258	34.355	-13.531	149	VAL	CA	-6.987	34.965	-12.269
149	VAL	C	-5.700	34.385	-11.613	149	VAL	O	-5.626	33.173	-11.639
149	VAL	CB	-6.224	34.890	-11.315	149	VAL	CG1	-7.893	35.619	-10.089
149	VAL	CG2	-9.456	35.386	-12.096	150	VAL	N	-4.732	35.301	-11.684
150	VAL	CA	-3.393	36.987	-10.903	150	VAL	C	-3.157	35.623	-9.559
150	VAL	O	-3.592	36.778	-9.400	150	VAL	CB	-2.276	35.385	-11.951
150	VAL	CG1	-0.973	34.633	-11.461	150	VAL	CG2	-2.673	34.843	-13.301
151	ALA	N	-2.568	36.946	-8.595	151	ALA	CA	-2.361	35.382	-7.287
151	ALA	C	-1.080	35.036	-6.657	151	ALA	O	-0.618	33.889	-6.986
151	ALA	CB	-3.557	35.390	-6.307	152	ALA	N	-0.690	35.987	-5.022
152	ALA	CA	0.714	35.638	-5.112	152	ALA	C	0.306	34.320	-4.188
152	ALA	O	-0.728	34.466	-3.467	152	ALA	CB	1.266	36.607	-4.296
153	ALA	N	1.125	33.302	-3.012	153	ALA	CA	0.860	32.250	-2.943
153	ALA	C	0.931	32.725	-1.811	153	ALA	O	0.317	32.192	-0.599
153	ALA	CB	1.750	31.038	-3.195	154	GLY	N	1.827	33.693	-1.266
154	GLY	CA	2.063	34.231	0.125	154	GLY	C	3.519	34.069	0.330
154	GLY	O	4.189	33.267	-0.118	155	ASN	N	3.958	34.788	1.968
155	ASN	CA	5.344	34.787	2.037	155	ASN	C	5.399	34.258	3.662
155	ASN	O	6.101	34.829	4.293	155	ASN	CB	6.808	36.198	1.906
155	ASN	CG	5.890	36.702	0.900	155	ASN	OD1	6.123	36.865	-0.536
155	ASN	ND2	5.656	37.963	0.352	156	GLU	N	4.711	33.168	3.675
156	GLU	CA	4.633	32.537	4.970	156	GLU	C	5.322	31.328	5.283
156	GLU	O	5.374	30.637	6.222	156	GLU	CB	3.205	31.980	5.180
156	GLU	CG	2.491	32.462	6.368	156	GLU	CD	2.396	33.951	6.270
156	GLU	OE1	1.744	34.322	5.312	156	GLU	OE2	3.186	34.656	7.146
157	GLY	N	6.389	31.057	4.227	157	GLY	CA	7.306	29.937	4.387
157	GLY	C	6.503	28.622	4.553	157	GLY	O	5.616	28.368	4.089
158	THR	N	7.147	27.793	5.302	158	THR	CG2	8.079	25.396	3.850
158	THR	OG1	8.707	25.487	6.217	158	THR	CB	7.864	25.346	5.296
158	THR	CA	6.952	26.487	5.702	158	THR	C	6.190	26.480	7.137
158	THR	O	6.479	27.335	7.977	159	SER	N	5.338	25.441	7.497
159	SER	OG	3.141	25.906	10.528	159	SER	CB	3.673	26.105	9.212
159	SER	CA	4.835	25.210	8.855	159	SER	C	4.496	23.720	8.946
159	SER	O	3.329	23.281	9.030	160	GLY	N	5.574	22.967	8.835
160	GLY	CA	5.636	21.504	8.895	160	GLY	C	4.378	21.065	7.738
160	GLY	O	4.800	21.326	6.555	161	SER	N	3.525	20.310	8.116
161	SER	CA	2.654	19.777	7.056	161	SER	C	1.477	20.788	6.786
161	SER	O	0.696	20.347	5.869	161	SER	CB	2.346	18.293	7.271
161	SER	OG	1.854	18.028	8.585	162	SER	N	1.303	21.061	7.459
162	SER	CA	0.167	22.725	7.113	162	SER	C	0.430	23.582	5.068
162	SER	O	1.533	23.840	5.394	162	SER	CB	-0.213	23.666	8.262
162	SER	OG	0.306	23.991	9.480	163	SER	N	-0.679	23.921	5.397
163	SER	CA	-0.611	24.750	3.990	163	SER	C	-0.661	26.377	4.513
163	SER	O	-1.078	26.548	5.504	163	SER	CB	-1.890	24.662	3.231
163	SER	OG	-1.992	25.718	2.331	164	THR	N	0.387	26.932	3.852
164	THR	CA	0.609	28.340	4.312	164	THR	C	0.185	29.286	3.196
164	THR	O	0.685	30.502	3.278	164	THR	CB	2.095	28.518	4.818
164	THR	OG1	2.986	28.282	3.692	164	THR	CG2	2.397	27.610	6.081
165	VAL	N	-0.513	28.742	2.190	165	VAL	CA	-0.959	29.962	1.010
165	VAL	C	-2.028	30.545	1.497	165	VAL	O	-2.929	30.132	2.280

165	VAL	CB	-1.339	28.024	-0.381	165	VAL	CG1	-1.947	29.357	-1.376
165	VAL	CG2	-3.210	27.736	-0.695	166	GLY	N	-1.910	31.021	1.129
166	GLY	CA	-2.943	32.778	1.620	166	GLY	C	-4.090	32.059	0.617
166	GLY	O	-4.126	32.386	-0.396	167	TYR	N	-5.056	33.130	0.970
167	TYR	CA	-6.223	34.066	0.113	167	TYR	C	-5.993	35.389	-0.688
167	TYR	O	-5.476	36.203	0.084	167	TYR	CB	-7.466	34.252	0.966
167	TYR	CG	-7.791	32.966	1.709	167	TYR	CD1	-7.208	32.703	2.947
167	TYR	CD2	-8.710	32.116	1.133	167	TYR	CE1	-7.367	31.328	3.618
167	TYR	CE2	-9.068	30.935	1.809	167	TYR	CZ	-8.486	30.671	3.046
167	TYR	OH	-8.800	29.481	3.030	168	PRO	N	-6.380	35.499	-1.830
168	PRO	CG	-6.943	36.376	-3.938	168	PRO	CD	-6.273	36.752	-2.626
168	PRO	CB	-7.966	35.366	-3.905	168	PRO	CA	-7.136	36.457	-2.560
168	PRO	C	-6.398	33.336	-3.270	168	PRO	O	-7.097	32.320	-3.912
169	GLY	N	-5.086	33.193	-3.189	169	GLY	CA	-4.446	32.077	-3.927
169	GLY	C	-4.937	30.702	-3.470	169	GLY	O	-4.080	29.733	-4.249
170	LYS	N	-5.402	30.579	-2.285	170	LYS	CA	-5.856	29.263	-1.745
170	LYS	C	-7.055	28.773	-2.516	170	LYS	O	-7.308	27.856	-2.826
170	LYS	CB	-6.246	29.296	-0.286	170	LYS	CG	-5.795	28.186	0.583
170	LYS	CD	-6.250	28.289	2.031	170	LYS	CE	-5.731	27.271	3.029
170	LYS	NZ	-6.259	27.463	3.215	171	TYR	N	-7.038	29.616	-3.168
171	TYR	CA	-5.012	29.063	-3.859	171	TYR	C	-8.683	28.389	-5.113
171	TYR	O	-7.760	28.714	-5.928	171	TYR	CB	-9.962	30.224	-4.262
171	TYR	CG	-10.497	30.864	-3.047	171	TYR	CD1	-11.060	30.303	-1.982
171	TYR	CD2	-10.456	32.374	-3.026	171	TYR	CE1	-11.520	31.003	-0.867
171	TYR	CE2	-10.961	33.088	-1.936	171	TYR	CZ	-11.528	32.398	-0.886
171	TYR	OH	-12.808	33.119	0.370	172	PRO	N	-9.297	27.206	-5.376
172	PRO	CA	-9.093	26.417	-6.396	172	PRO	C	-9.233	27.156	-7.909
172	PRO	O	-8.325	26.704	-8.881	172	PRO	CB	-10.167	25.329	-6.513
172	PRO	CG	-10.630	29.271	-8.096	172	PRO	CD	-10.364	26.669	-4.816
173	SER	N	-10.897	28.167	-8.019	173	SER	CA	-10.220	28.018	-9.330
173	SER	C	-9.025	29.773	-9.895	173	SER	O	-8.966	30.233	-10.742
173	SER	CB	-11.528	29.623	-9.481	173	SER	OG	-11.595	30.566	-8.406
174	VAL	N	-8.162	29.944	-8.626	174	VAL	CA	-7.053	30.891	-8.855
174	VAL	C	-5.756	30.131	-9.068	174	VAL	O	-5.612	29.132	-8.366
174	VAL	CB	-6.899	31.775	-7.596	174	VAL	CG1	-5.796	32.837	-7.617
174	VAL	CG2	-8.220	32.303	-7.323	175	ILE	N	-4.911	30.729	-9.885
175	ILE	CA	-3.869	30.156	-10.024	175	ILE	C	-2.716	30.736	-8.896
175	ILE	O	-2.450	31.958	-8.955	175	ILE	CB	-2.953	30.526	-11.419
175	ILE	CG1	-3.857	29.978	-12.524	175	ILE	CG2	-1.481	30.019	-11.812
175	ILE	CD1	-3.692	30.529	-13.946	176	ALA	N	-2.220	30.028	-7.925
176	ALA	CA	-1.335	30.517	-6.870	176	ALA	C	0.120	30.301	-7.310
176	ALA	O	0.453	29.238	-7.838	176	ALA	CB	-1.639	29.838	-8.563
177	VAL	N	0.064	31.430	-7.180	177	VAL	CA	2.261	31.836	-7.636
177	VAL	C	3.225	31.693	-6.473	177	VAL	O	3.170	32.657	-5.721
177	VAL	CB	2.439	32.607	-8.755	177	VAL	CG1	3.062	32.667	-9.392
177	VAL	CG2	1.376	32.852	-9.845	178	GLY	N	4.877	30.656	-6.358
178	GLY	CA	5.160	30.703	-5.339	178	GLY	C	6.646	31.233	-6.076
178	GLY	O	6.499	31.638	-7.286	179	ALA	N	7.812	31.467	-5.287
179	ALA	CA	8.715	32.037	-5.859	179	ALA	C	9.939	31.099	-5.779
179	ALA	C	10.193	30.681	-4.719	179	ALA	CB	9.025	33.251	-4.973
180	VAL	N	10.639	31.162	-6.885	180	VAL	CA	11.970	30.472	-6.981
180	VAL	C	13.945	31.585	-7.171	180	VAL	O	12.712	32.691	-7.627
180	VAL	CB	12.078	29.514	-8.166	180	VAL	CG1	11.271	28.251	-7.858
180	VAL	CG2	11.673	30.129	-9.500	181	ASP	N	14.267	31.203	-6.800
181	ASP	CA	15.631	32.108	-7.039	181	ASP	C	15.942	31.804	-8.662
181	ASP	O	15.339	31.090	-9.292	181	ASP	CB	16.666	31.921	-5.916
181	ASP	CG	17.120	30.536	-5.971	181	ASP	OD1	17.103	29.703	-6.972
181	ASP	OD2	17.600	30.286	-4.887	182	SER	N	17.087	32.386	-8.047
182	SER	CA	17.622	32.216	-10.191	182	SER	C	18.153	30.817	-10.496
182	SER	O	18.365	30.452	-11.670	182	SER	CB	18.678	33.313	-10.666
182	SER	OG	18.016	34.961	-10.475	183	SER	N	18.258	30.962	-9.623
183	SER	CA	18.716	28.665	-9.666	183	SER	C	17.351	27.616	-9.947
183	SER	O	17.859	26.615	-9.397	183	SER	CB	19.256	28.323	-8.807

183	SER OG	15.589	28.615	-8.251	184	ASN N	16.373	28.094	-9.682
184	ASN CA	13.144	27.317	-9.580	184	ASN C	14.931	26.720	-8.197
184	ASN O	14.138	25.759	-8.007	184	ASN CB	15.014	26.341	-10.722
184	ASN CG	16.993	26.908	-12.076	184	ASN OD1	14.780	25.106	-11.277
184	ASN ND2	15.352	26.210	-13.076	185	GLN N	13.942	27.247	-7.159
185	GLN CA	15.270	26.646	-5.035	185	GLN C	14.280	27.494	-5.283
185	GLN O	14.159	28.726	-5.316	185	GLN CB	16.599	26.968	-5.101
185	GLN CG	16.539	26.242	-3.614	185	GLN CD	18.011	26.102	-3.296
185	GLN OE1	18.864	25.799	-4.861	185	GLN NE2	18.266	26.386	-1.936
186	ARG N	13.278	26.931	-4.448	186	ARG CA	12.185	27.774	-3.841
186	ARG C	12.780	28.782	-2.066	186	ARG O	13.698	28.386	-2.093
186	ARG CB	11.215	26.843	-3.116	186	ARG CG	10.216	27.671	-2.161
186	ARG CD	9.467	26.337	-1.468	186	ARG NE	9.066	26.333	-0.117
186	ARG CZ	9.962	26.879	1.059	186	ARG NH1	9.367	27.880	1.658
186	ARG NH2	10.966	26.321	1.783	187	ALA N	12.294	30.089	-2.033
187	ALA CA	12.728	31.066	-1.095	187	ALA C	12.262	30.604	-0.517
187	ALA O	11.158	30.043	-0.387	187	ALA CB	12.166	32.402	-2.366
188	SER N	13.051	30.770	0.549	188	SER CA	12.673	30.286	1.068
188	SER C	11.356	30.847	2.412	188	SER O	10.740	30.311	3.212
188	SER CB	13.767	30.456	2.938	188	SER OG	14.137	31.026	2.041
189	PHE N	10.963	32.010	1.974	189	PHE CA	9.697	32.680	2.618
189	PHE C	8.499	32.198	1.609	189	PHE O	7.389	32.856	2.011
189	PHE CB	9.787	34.217	2.263	189	PHE CG	10.117	34.698	0.867
189	PHE CD1	9.167	34.830	-0.121	189	PHE CD2	11.615	35.316	0.967
189	PHE CE1	9.633	35.187	-1.411	189	PHE CE2	11.769	35.945	-0.781
189	PHE CZ	10.786	35.586	-1.725	190	SER N	8.703	31.326	0.491
190	SER CA	7.626	31.096	-0.391	190	SER C	6.663	30.162	0.328
190	SER O	7.834	29.083	0.066	190	SER CB	8.131	30.390	-1.788
190	SER OG	7.136	30.337	-2.618	191	SER N	5.308	30.951	0.326
191	SER CA	4.341	29.676	0.987	191	SER C	4.261	28.330	0.223
191	SER O	4.543	28.268	-0.995	191	SER CB	3.015	30.411	0.911
191	SER OG	2.729	31.285	1.954	192	VAL N	3.756	27.310	0.928
192	VAL CA	3.627	25.932	0.391	192	VAL C	2.254	25.291	0.686
192	VAL O	1.559	25.698	1.598	192	VAL CB	4.781	25.127	1.981
192	VAL CG1	6.144	25.727	0.722	192	VAL CG2	6.617	25.106	2.592
193	GLY N	1.938	24.372	0.047	193	GLY CA	0.629	23.964	0.610
193	GLY C	0.081	23.029	-0.901	193	GLY O	0.530	23.246	-2.015
194	PRO N	-1.023	22.289	-0.722	194	PRO CA	-1.662	21.651	-1.873
194	PRO C	-2.237	22.605	-2.934	194	PRO O	-2.603	22.246	-4.081
194	PRO CB	-2.769	20.783	-1.210	194	PRO CG	-2.311	20.622	0.213
194	PRO CD	-1.633	21.956	0.578	195	GLU N	-2.322	23.793	-2.639
195	GLU CA	-3.168	24.850	-3.282	195	GLU C	-2.095	25.631	-4.051
195	GLU O	-2.516	26.398	-4.936	195	GLU CB	-4.063	25.786	-2.670
195	GLU CG	-4.942	25.136	-1.635	195	GLU CD	-4.315	24.860	-0.100
195	GLU OE1	-3.110	24.960	0.165	195	GLU OE2	-5.130	24.520	0.785
196	LEU N	-0.829	25.264	-3.870	196	LEU CA	0.241	25.929	-4.666
196	LEU C	0.228	25.376	-6.059	196	LEU O	0.305	24.121	-6.153
196	LEU CB	1.360	25.739	-3.854	196	LEU CG	2.770	26.178	-4.643
196	LEU CD1	2.739	27.716	-4.639	196	LEU CD2	4.027	25.721	-3.911
197	ASP N	0.140	26.208	-7.093	197	ASP CA	0.932	25.774	-8.480
197	ASP C	1.307	25.738	-9.293	197	ASP O	1.055	24.736	-9.916
197	ASP CB	-1.067	26.598	-9.191	197	ASP CG	-2.406	26.351	-8.569
197	ASP OD1	-2.804	25.155	-8.394	197	ASP OD2	-3.035	27.327	-8.008
198	VAL N	2.013	26.889	-9.344	198	VAL CA	3.206	26.970	-10.209
198	VAL C	4.157	27.050	-9.516	198	VAL O	3.752	28.099	-8.887
198	VAL CB	2.596	27.476	-11.637	198	VAL CG1	1.930	26.726	-12.937
198	VAL CG2	2.337	28.919	-11.686	199	MET N	5.374	27.916	-10.816
199	MET CA	6.439	28.802	-9.498	199	MET C	6.845	29.810	-10.578
199	MET O	6.696	29.518	-11.793	199	MET CB	7.660	27.970	-9.077
199	MET CG	7.363	26.869	-8.139	199	MET SD	6.783	27.449	-6.568
199	MET CE	8.227	27.755	-5.387	200	ALA N	7.426	30.962	-10.303
200	ALA CA	7.991	31.929	-11.035	200	ALA C	9.088	32.066	-10.272
200	ALA O	8.127	32.026	-9.860	200	ALA CB	6.932	32.078	-11.630

201	PRO	N	9.027	38.499	-10.981	201	PRO	CA	11.013	34.130	-10.238
201	PRO	C	10.450	35.127	-9.231	201	PRO	O	9.579	35.987	-9.682
201	PRO	CB	11.017	34.723	-11.400	201	PRO	CG	11.392	34.040	-12.678
201	PRO	CD	9.961	33.616	-12.405	202	GLY	N	10.925	35.286	-8.021
202	GLY	CA	10.473	36.236	-7.846	202	GLY	C	11.380	36.698	-6.315
202	GLY	O	11.352	37.126	-4.979	203	VAL	N	12.815	36.503	-6.613
203	VAL	CA	13.948	36.929	-5.716	203	VAL	C	14.706	38.017	-6.469
203	VAL	C	15.133	37.731	-7.593	203	VAL	CB	16.016	35.688	-5.391
203	VAL	CG1	16.096	36.106	-6.632	203	VAL	CG2	14.879	34.743	-4.398
204	SER	N	14.865	39.102	-5.339	204	SER	CA	15.572	40.281	-6.487
204	SER	C	15.067	40.619	-7.872	204	SER	O	15.706	40.681	-8.889
204	SER	CB	17.087	39.976	-6.326	204	SER	OG	17.752	41.188	-6.672
205	ILE	N	13.771	40.965	-8.008	205	ILE	CA	13.869	42.236	-9.225
205	ILE	C	13.207	42.769	-9.470	205	ILE	O	12.675	43.698	-8.648
205	ILE	CB	11.832	40.833	-9.144	205	ILE	CG1	11.436	39.336	-8.010
205	ILE	CG2	10.899	41.281	-10.467	205	ILE	CD1	12.257	38.412	-9.771
206	GLN	N	13.956	43.995	-10.489	206	GLN	CA	14.204	44.517	-10.834
206	GLN	C	13.802	44.978	-11.630	206	GLN	O	12.669	44.318	-12.621
206	GLN	CB	15.455	44.705	-11.740	206	GLN	CG	16.684	44.163	-10.980
206	GLN	CD	17.205	45.168	-10.007	206	GLN	OE1	18.328	44.936	-9.353
206	GLN	NE2	16.556	46.260	-9.857	207	SER	N	12.359	46.866	-11.214
207	SER	CA	11.217	46.571	-11.987	207	SER	C	11.089	48.093	-11.749
207	SER	O	11.919	48.657	-11.004	207	SER	CB	9.918	45.833	-11.869
207	SER	OG	8.993	46.056	-12.613	208	THR	N	10.856	48.666	-12.326
208	THR	CG2	9.171	50.339	-14.754	208	THR	OG1	7.570	49.414	-13.166
208	THR	CB	8.620	50.415	-13.357	208	THR	CA	9.675	50.892	-12.173
208	THR	C	9.197	50.488	-10.803	208	THR	O	8.423	49.807	-10.869
209	LEU	N	9.656	51.613	-10.228	209	LEU	CA	9.192	52.158	-8.959
209	LEU	C	8.673	53.610	-9.262	209	LEU	O	9.160	54.227	-10.222
209	LEU	CB	10.335	52.192	-7.958	209	LEU	CG	10.806	55.816	-7.616
209	LEU	CD1	11.968	51.316	-6.472	209	LEU	CD2	9.607	50.282	-6.669
210	PRO	N	7.790	54.339	-8.444	210	PRO	CA	7.273	55.517	-8.649
210	PRO	C	8.383	56.573	-8.639	210	PRO	O	9.491	56.663	-8.106
210	PRO	CB	6.302	55.733	-7.517	210	PRO	CG	6.004	54.379	-6.966
210	PRO	CD	7.193	53.693	-7.271	211	GLY	N	8.077	57.668	-9.355
211	GLY	CA	9.069	58.765	-9.610	211	GLY	C	10.094	58.656	-10.498
211	GLY	O	11.176	59.005	-10.259	212	ASN	N	9.881	57.770	-11.887
212	ASN	CA	10.903	57.622	-12.643	212	ASN	C	12.039	56.753	-12.096
212	ASN	C	13.188	57.381	-12.420	212	ASN	CB	11.224	58.393	-13.499
212	ASN	CG	11.803	58.185	-14.836	212	ASN	OD1	11.853	57.856	-15.323
212	ASN	ND2	12.273	59.159	-15.376	213	LYS	N	11.803	55.769	-11.247
213	LYS	CA	12.810	54.946	-10.537	213	LYS	C	12.668	53.659	-10.866
213	LYS	O	11.775	53.039	-11.613	213	LYS	CB	12.769	55.241	-9.859
213	LYS	CG	13.206	56.694	-8.767	213	LYS	CD	13.246	57.830	-7.312
213	LYS	CE	14.195	58.218	-6.870	213	LYS	NZ	15.048	58.705	-7.921
214	TYR	N	13.681	52.703	-10.444	214	TYR	CA	13.803	51.246	-10.722
214	TYR	C	14.383	50.600	-9.489	214	TYR	O	15.211	51.253	-8.817
214	TYR	CB	14.661	50.981	-11.984	214	TYR	CG	14.130	51.621	-13.246
214	TYR	CD1	14.689	52.047	-13.678	214	TYR	CD2	13.129	51.068	-14.816
214	TYR	CE1	14.230	53.475	-14.816	214	TYR	CE2	12.656	51.669	-15.178
214	TYR	CZ	13.204	52.895	-15.850	214	TYR	OH	12.756	53.658	-16.696
215	GLY	N	14.838	49.847	-9.158	215	GLY	CA	14.622	48.772	-7.905
215	GLY	C	14.130	47.329	-7.769	215	GLY	O	13.249	46.917	-8.821
216	ALA	N	14.810	46.610	-6.831	216	ALA	CA	14.654	45.203	-6.781
216	ALA	C	13.682	44.922	-5.512	216	ALA	O	13.948	45.527	-4.478
216	ALA	CB	15.715	44.356	-6.887	217	TYR	N	12.788	43.982	-5.875
217	TYR	CA	11.964	43.688	-4.440	217	TYR	C	12.833	41.928	-4.567
217	TYR	O	12.262	41.642	-5.656	217	TYR	CB	10.473	43.862	-4.570
217	TYR	CG	10.117	43.291	-4.216	217	TYR	CD1	10.846	45.991	-3.236
217	TYR	CD2	9.016	45.933	-6.785	217	TYR	CE1	10.459	47.267	-2.790
217	TYR	CE2	8.654	47.219	-6.381	217	TYR	CZ	9.358	47.882	-3.391
217	TYR	OH	8.953	49.160	-2.980	218	ASN	N	11.750	41.308	-3.391
218	ASN	CA	11.640	39.042	-3.227	218	ASN	C	10.286	38.636	-2.769

218	ASN	O	9.763	43.367	-1.017	218	ASN	CB	12.959	39.360	-5.136
218	ASN	CG	16.031	39.366	-2.363	218	ASN	OD1	16.612	39.709	-3.622
218	ASN	ND2	16.660	39.644	-3.165	219	GLY	N	9.670	38.654	-2.289
219	GLY	CA	8.382	38.132	-2.649	219	GLY	C	7.570	37.386	-3.681
219	GLY	O	7.873	37.802	-4.876	220	THR	N	6.563	36.638	-3.203
220	THR	CA	8.697	35.936	-4.179	220	THR	C	6.879	37.064	-6.866
220	THR	C	6.617	36.762	-5.938	220	THR	CB	6.825	36.019	-3.926
220	THR	OG1	6.136	35.363	-2.491	220	THR	CG2	5.704	33.096	-2.980
221	SER	N	4.738	38.238	-4.303	221	SER	CA	3.984	39.201	-5.169
221	SER	C	6.760	39.661	-6.383	221	SER	O	6.117	40.208	-7.277
221	SER	CB	3.323	40.383	-4.846	221	SER	OG	3.635	40.282	-3.160
222	MET	N	6.063	39.389	-6.685	222	MET	CE	6.671	42.771	-8.173
222	MET	SD	7.768	41.333	-6.993	222	MET	CG	8.506	41.399	-6.602
222	MET	CB	8.351	40.023	-7.218	222	MET	CA	6.916	39.670	-7.638
222	MET	C	6.877	38.633	-8.367	222	MET	O	7.084	38.867	-9.775
223	ALA	N	6.556	37.246	-8.061	223	ALA	CA	6.469	36.020	-8.885
223	ALA	C	5.200	36.068	-9.707	223	ALA	O	5.133	35.968	-10.929
223	ALA	CB	6.509	34.807	-7.923	224	SER	N	4.070	36.360	-9.838
224	SER	CA	2.738	36.488	-9.703	224	SER	C	2.661	37.161	-11.039
224	SER	O	2.165	36.593	-12.097	224	SER	CB	1.801	36.991	-8.603
224	SER	OG	8.692	36.895	-9.397	225	PRO	N	3.156	38.611	-11.159
225	PRO	CA	3.895	39.130	-12.639	225	PRO	C	3.764	38.469	-13.626
225	PRO	O	3.406	38.650	-14.804	225	PRO	CB	3.653	40.811	-12.836
225	PRO	CG	6.411	40.402	-10.764	225	PRO	CD	3.733	39.226	-10.856
226	HIS	N	4.769	37.626	-13.299	226	HIS	CA	5.468	36.079	-14.862
226	HIS	C	4.618	35.947	-15.061	226	HIS	O	4.625	38.869	-16.293
226	HIS	CB	6.608	36.046	-13.765	226	HIS	CG	7.816	36.839	-13.358
226	HIS	ND1	8.048	37.488	-12.170	226	HIS	CD2	8.883	37.118	-14.167
226	HIS	CE1	9.270	38.052	-12.236	226	HIS	NE2	9.771	37.866	-13.663
227	VAL	N	3.593	35.366	-14.199	227	VAL	CA	2.883	34.388	-16.727
227	VAL	C	1.679	35.197	-15.621	227	VAL	O	1.018	34.773	-16.690
227	VAL	CB	2.203	33.644	-13.619	227	VAL	CG1	1.076	32.676	-14.266
227	VAL	CG2	3.204	32.668	-12.893	228	ALA	N	1.003	36.242	-14.816
228	ALA	CA	0.031	37.109	-15.517	228	ALA	C	0.563	37.538	-16.868
228	ALA	O	-0.253	37.633	-17.828	228	ALA	CB	-0.307	38.333	-14.668
229	GLY	N	1.791	38.028	-16.963	229	GLY	CA	2.352	38.408	-18.239
229	GLY	C	2.420	37.197	-19.187	229	GLY	O	2.189	37.375	-20.384
230	ALA	N	2.711	35.988	-16.666	230	ALA	CA	2.794	36.801	-19.866
230	ALA	C	1.424	36.800	-20.153	230	ALA	O	1.380	36.269	-21.363
230	ALA	CB	3.298	33.624	-18.709	231	ALA	N	0.385	36.623	-19.328
231	ALA	CA	-1.010	34.616	-19.764	231	ALA	C	-1.256	35.423	-20.864
231	ALA	O	-1.909	35.056	-21.852	231	ALA	CB	-1.932	34.666	-18.869
232	ALA	N	-0.778	36.657	-20.721	232	ALA	CA	-1.013	37.663	-21.792
232	ALA	C	-0.281	37.284	-23.078	232	ALA	O	-0.863	37.901	-24.187
232	ALA	CB	-0.742	39.121	-21.377	233	LEU	N	0.935	36.726	-22.987
233	LEU	CA	1.627	36.293	-24.209	233	LEU	C	0.821	35.169	-24.880
233	LEU	O	0.696	35.231	-26.111	233	LEU	CB	3.063	35.877	-23.907
233	LEU	CG	3.996	36.994	-23.653	233	LEU	CD1	5.239	38.362	-22.921
233	LEU	CD2	4.241	37.833	-26.680	234	ILE	N	0.337	36.399	-24.067
234	ILE	CD1	0.306	30.666	-21.637	234	ILE	CG1	0.656	31.223	-23.103
234	ILE	CB	-0.811	32.016	-23.570	234	ILE	CG2	-1.803	30.900	-24.891
234	ILE	CA	-0.406	33.076	-24.646	234	ILE	C	-1.621	33.997	-25.636
234	ILE	O	-1.883	33.166	-26.566	235	LEU	N	-2.390	36.665	-24.779
235	LEU	CA	-3.596	35.028	-25.623	235	LEU	C	-3.258	38.863	-26.672
235	LEU	O	-4.109	35.916	-27.589	235	LEU	CB	-6.632	35.769	-26.378
235	LEU	CG	-5.160	34.899	-23.362	235	LEU	CD1	-5.652	35.683	-22.165
235	LEU	CD2	-6.282	34.138	-24.120	236	SER	N	-2.096	36.638	-26.798
236	SER	CA	-1.764	37.237	-27.986	236	SER	C	-1.691	36.292	-29.166
236	SER	O	-1.746	36.636	-30.290	236	SER	CB	-0.633	38.236	-27.733
236	SER	OG	0.599	37.571	-27.582	237	LYS	N	-1.866	33.067	-28.882
237	LYS	CA	-0.868	34.033	-29.952	237	LYS	C	-2.113	33.277	-30.268
237	LYS	O	-2.378	32.953	-31.444	237	LYS	CB	0.272	33.112	-29.393
237	LYS	CG	0.677	32.260	-30.716	237	LYS	CD	2.020	31.335	-30.662

237	LYS	CE	2.345	30.762	-31.720
238	HIS	N	-2.931	31.989	-29.312
238	HIS	C	-5.334	32.899	-28.697
238	HIS	CB	-3.968	30.862	-28.311
238	HIS	ND1	-1.707	29.670	-28.835
238	HIS	CE1	-1.886	28.831	-29.642
239	PRO	N	-5.848	33.937	-29.348
239	PRO	C	-8.206	34.252	-28.332
239	PRO	CB	-7.818	35.977	-29.713
239	PRO	CD	-5.436	34.439	-30.668
240	ASN	CA	-9.329	32.041	-29.216
240	ASN	O	-10.360	30.610	-27.576
240	ASN	CG	-7.972	30.827	-30.889
240	ASN	ND2	-7.676	29.309	-30.926
241	TRP	CA	-8.304	30.126	-26.120
241	TRP	O	-9.043	31.833	-26.686
241	TRP	CG	-6.094	28.903	-26.357
241	TRP	CD2	-6.839	28.326	-26.185
241	TRP	CE2	-6.616	27.476	-27.210
241	TRP	CZ2	-3.195	26.786	-27.176
241	TRP	CH2	-2.670	26.873	-26.008
242	THR	CA	-10.658	30.139	-22.911
242	THR	O	-8.335	29.674	-21.937
242	THR	OG1	-10.837	27.786	-22.476
243	ASN	N	-9.966	30.639	-20.611
243	ASN	OD1	-11.465	31.518	-18.768
243	ASN	CB	-9.708	31.330	-18.332
243	ASN	C	-8.617	29.303	-19.010
244	THR	N	-9.566	28.362	-19.283
244	THR	C	-8.133	26.393	-19.802
244	THR	CB	-10.615	26.088	-19.494
244	THR	CG2	-10.533	24.595	-19.159
245	GLN	CA	-6.966	26.362	-21.962
245	GLN	O	-6.573	26.393	-21.647
245	GLN	CG	-8.265	25.526	-23.989
245	GLN	OE1	-9.306	26.769	-25.727
246	VAL	N	-5.697	28.306	-21.318
246	VAL	C	-3.936	26.682	-19.667
246	VAL	CB	-6.779	30.555	-20.621
246	VAL	CG2	-5.169	31.238	-21.959
247	ARG	CA	-4.380	27.734	-17.360
247	ARG	O	-2.705	25.985	-16.764
247	ARG	CG	-4.987	27.095	-14.832
247	ARG	NE	-5.640	26.757	-12.846
247	ARG	NH1	-7.066	27.686	-11.230
248	SER	N	-4.680	25.805	-18.331
248	SER	C	-2.657	24.086	-19.072
248	SER	CB	-5.036	23.408	-19.372
249	SER	N	-2.300	24.853	-20.136
249	SER	C	-0.071	25.302	-19.968
249	SER	CB	-1.369	25.758	-22.068
250	LEU	N	-0.289	26.333	-19.160
250	LEU	CD1	-0.373	30.433	-17.260
250	LEU	CB	0.170	28.063	-17.985
250	LEU	C	1.092	25.694	-17.265
251	GLN	N	0.068	25.867	-16.716
251	GLN	OE1	-2.819	23.626	-12.935
251	GLN	CG	-1.210	24.816	-13.996
251	GLN	CA	0.381	23.961	-13.765
251	GLN	O	1.743	22.816	-15.616
252	ASN	CA	1.882	21.204	-18.202
252	ASN	O	2.809	20.442	-19.768
252	ASN	CG	-1.036	19.926	-18.573
237	LYS	NZ	3.828	29.848	-31.996
238	HIS	CA	-4.360	32.163	-29.379
238	HIS	O	-5.713	32.886	-27.562
238	HIS	CG	-3.080	30.921	-29.237
238	HIS	CD2	-3.137	29.290	-30.396
238	HIS	NE2	-1.968	28.600	-30.399
239	PRO	CA	-6.988	34.779	-28.773
239	PRO	O	-8.969	34.819	-27.662
239	PRO	CG	-6.666	35.296	-31.827
240	ASN	N	-8.386	32.969	-29.227
240	ASN	C	-9.508	31.380	-27.980
240	ASN	CB	-9.603	31.269	-30.538
240	ASN	OD1	-7.898	31.390	-31.167
241	TRP	N	-8.356	31.006	-27.306
241	TRP	C	-9.106	30.638	-24.936
241	TRP	CB	-6.879	29.030	-25.679
241	TRP	CD1	-6.338	28.433	-27.818
241	TRP	NE1	-5.362	27.567	-28.231
241	TRP	CE3	-6.097	28.486	-24.981
241	TRP	CZ3	-2.912	27.667	-24.963
242	THR	N	-8.727	29.781	-24.162
242	THR	C	-9.469	30.176	-21.767
242	THR	CB	-11.579	29.032	-22.678
242	THR	CG2	-12.696	28.907	-23.098
243	ASN	ND2	-11.787	30.686	-18.767
243	ASN	CG	-11.093	31.331	-17.985
243	ASN	CA	-9.853	30.731	-19.666
243	ASN	O	-7.893	29.136	-18.660
244	THR	CA	-9.381	26.936	-19.859
244	THR	O	-7.326	25.787	-19.111
244	THR	OG1	-11.738	26.675	-18.686
245	GLN	N	-8.082	26.716	-21.073
245	GLN	C	-5.647	27.020	-21.520
245	GLN	CB	-7.330	26.899	-23.297
245	GLN	CD	-8.693	25.873	-25.628
245	GLN	NE2	-7.765	25.332	-26.370
246	VAL	CA	-6.677	29.060	-20.778
246	VAL	O	-2.708	28.227	-19.361
246	VAL	CG1	-3.866	31.272	-20.827
247	ARG	N	-6.767	28.260	-18.662
247	ARG	C	-3.770	26.292	-17.360
247	ARG	CB	-3.533	27.667	-16.169
247	ARG	CD	-6.856	27.179	-13.793
247	ARG	CZ	-5.893	26.066	-11.313
247	ARG	NH2	-5.177	26.628	-10.270
248	SER	CA	-6.839	24.131	-18.626
248	SER	O	-1.848	23.283	-18.883
248	SER	OG	-6.146	23.890	-18.832
249	SER	CA	-1.223	24.874	-20.851
249	SER	O	1.826	24.785	-20.069
249	SER	OG	-0.308	25.619	-22.956
250	LEU	CD2	1.824	29.036	-18.222
250	LEU	CG	0.352	29.638	-18.151
250	LEU	CA	0.718	26.837	-18.216
250	LEU	C	2.283	25.621	-17.032
251	GLN	NE2	-2.750	25.332	-12.237
251	GLN	CD	-2.365	24.350	-13.036
251	GLN	CB	-0.887	23.621	-14.877
251	GLN	C	0.919	22.664	-16.361
252	ASN	N	0.633	22.396	-17.390
252	ASN	C	2.396	21.389	-18.991
252	ASN	CB	0.806	20.780	-19.292
252	ASN	OD1	-0.836	19.385	-17.882

252	ASN	ND2	-2.234	19.934	-19.361	253	THR	N	5.818	22.805	-18.921
253	THR	CA	6.256	22.717	-19.715	253	THR	C	5.381	23.247	-18.818
253	THR	O	6.365	25.733	-19.627	253	THR	CB	4.986	23.672	-20.952
253	THR	OG1	3.595	24.957	-20.428	253	THR	CG2	3.547	23.130	-22.032
254	THR	N	8.218	23.177	-17.551	254	THR	CA	6.216	23.612	-16.588
254	THR	C	7.466	22.730	-16.612	254	THR	O	7.402	21.980	-17.093
254	THR	CB	5.664	23.958	-15.132	254	THR	OG1	5.129	22.178	-15.040
254	THR	CG2	4.530	24.549	-14.802	255	THR	N	8.499	23.286	-16.876
255	THR	CA	9.771	22.594	-15.817	255	THR	C	9.621	22.031	-14.414
255	THR	O	9.439	22.786	-13.474	255	THR	CB	11.080	23.455	-15.897
255	THR	OG1	11.982	23.709	-17.321	255	THR	CG2	12.286	22.620	-15.406
256	LYS	N	9.606	20.702	-14.314	256	LYS	CA	9.364	20.063	-13.018
256	LYS	C	10.322	20.333	-12.063	256	LYS	O	11.662	20.274	-12.592
256	LYS	CB	9.026	18.590	-13.249	256	LYS	CG	9.818	17.805	-11.921
256	LYS	CD	10.286	16.948	-11.777	256	LYS	CE	10.232	15.940	-10.623
256	LYS	NZ	9.263	14.969	-11.056	257	LEU	N	10.212	20.674	-10.824
257	LEU	CA	11.272	21.035	-9.893	257	LEU	C	11.230	20.232	-8.614
257	LEU	O	12.096	20.865	-7.732	257	LEU	CB	11.387	22.547	-9.322
257	LEU	CG	11.357	23.620	-10.968	257	LEU	CD1	11.268	25.003	-9.921
257	LEU	CD2	12.678	23.468	-11.325	258	GLY	N	10.431	19.282	-8.288
258	GLY	CA	10.602	18.793	-6.879	258	GLY	C	9.168	18.703	-6.373
258	GLY	O	8.283	18.956	-7.202	259	ASP	N	9.026	18.282	-5.180
259	ASP	CA	7.757	17.896	-4.516	259	ASP	C	6.619	18.941	-4.789
259	ASP	O	4.859	20.039	-4.214	259	ASP	CB	7.994	17.960	-3.033
259	ASP	CG	6.781	17.128	-2.243	259	ASP	OD1	5.611	17.327	-2.354
259	ASP	OD2	7.058	16.299	-1.321	260	SER	N	5.560	18.610	-5.312
260	SER	CA	6.483	19.587	-5.529	260	SER	C	4.946	20.342	-6.289
260	SER	O	3.500	21.523	-4.646	260	SER	CB	3.345	18.919	-6.289
260	SER	OG	2.745	17.937	-5.468	261	PHE	N	4.261	19.778	-3.112
261	PHE	CA	3.831	20.468	-1.085	261	PHE	C	4.544	21.866	-1.863
261	PHE	O	3.944	22.848	-1.432	261	PHE	CB	4.053	19.749	-0.563
261	PHE	CG	3.549	20.337	0.715	261	PHE	CD1	2.206	20.163	1.123
261	PHE	CD2	4.493	21.060	1.558	261	PHE	CE1	1.737	20.717	2.315
261	PHE	CE2	3.963	21.802	2.748	261	PHE	CZ	2.605	21.465	3.114
262	TYR	N	5.778	21.788	-2.305	262	TYR	CA	6.688	22.916	-2.251
262	TYR	C	6.820	23.619	-3.545	262	TYR	O	7.201	24.953	-3.393
262	TYR	CB	8.122	22.435	-1.831	262	TYR	CG	8.146	21.892	-0.456
262	TYR	CD1	8.084	20.484	-0.364	262	TYR	CD2	8.149	22.669	0.698
262	TYR	CE1	8.062	19.873	0.882	262	TYR	CE2	8.114	22.069	1.962
262	TYR	CZ	8.069	20.672	2.018	262	TYR	OH	7.965	20.029	3.205
263	TYR	N	6.626	23.106	-6.693	263	TYR	CA	6.812	23.655	-6.822
263	TYR	C	8.626	23.680	-6.956	263	TYR	O	5.781	24.117	-8.111
263	TYR	CB	7.928	22.768	-6.681	263	TYR	CG	9.279	23.035	-6.068
263	TYR	CD1	10.066	24.046	-6.637	263	TYR	CD2	9.805	23.342	-4.995
263	TYR	CE1	11.335	24.328	-6.168	263	TYR	CE2	11.062	22.660	-4.491
263	TYR	CZ	11.838	23.618	-5.186	263	TYR	OH	13.063	23.969	-4.897
264	GLY	N	4.673	23.163	-6.518	264	GLY	CA	3.301	23.066	-7.612
264	GLY	C	3.847	22.196	-8.556	264	GLY	O	4.647	21.274	-8.365
265	LYS	N	3.636	22.477	-8.736	265	LYS	CA	3.834	21.798	-10.971
265	LYS	C	5.388	22.232	-11.464	265	LYS	O	5.616	21.863	-12.386
265	LYS	CB	2.755	22.071	-12.044	265	LYS	CG	1.690	21.863	-11.305
265	LYS	CD	0.710	20.568	-12.079	265	LYS	CE	-0.692	20.496	-11.391
265	LYS	NZ	-1.678	20.757	-12.489	266	GLY	N	5.787	23.226	-10.017
266	GLY	CA	7.120	23.612	-11.323	266	GLY	C	7.135	25.032	-11.818
266	GLY	O	6.177	25.793	-11.648	267	LEU	N	8.262	25.336	-12.480
267	LEU	CA	8.490	26.660	-13.097	267	LEU	C	7.804	26.771	-16.437
267	LEU	O	7.953	25.909	-15.298	267	LEU	CB	10.010	26.855	-13.214
267	LEU	CG	10.432	28.060	-14.058	267	LEU	CD1	10.896	29.331	-13.250
267	LEU	CD2	11.924	27.921	-16.327	268	ILE	N	7.064	27.063	-16.632
268	ILE	CA	6.406	28.035	-18.944	268	ILE	C	7.436	28.246	-17.065
268	ILE	O	8.539	28.793	-16.912	268	ILE	CB	8.369	29.210	-15.099
268	ILE	CG1	6.099	30.541	-15.552	268	ILE	CG2	6.263	28.925	-14.867
268	ILE	CD1	8.399	31.765	-16.262	269	ASN	N	7.887	27.843	-18.237

269	ASN	CA	[illegible]	[illegible]	[illegible]	269	ASN	C	[illegible]	[illegible]	[illegible]
269	ASN	O	[illegible]	[illegible]	[illegible]	269	ASN	CB	[illegible]	[illegible]	[illegible]
269	ASN	CG	[illegible]	[illegible]	[illegible]	269	ASN	OD1	[illegible]	[illegible]	[illegible]
269	ASN	ND2	[illegible]	[illegible]	[illegible]	270	VAL	N	[illegible]	[illegible]	[illegible]
270	VAL	CA	[illegible]	[illegible]	[illegible]	270	VAL	C	[illegible]	[illegible]	[illegible]
270	VAL	O	[illegible]	[illegible]	[illegible]	270	VAL	CB	[illegible]	[illegible]	[illegible]
270	VAL	CG1	[illegible]	[illegible]	[illegible]	270	VAL	CG2	[illegible]	[illegible]	[illegible]
271	GLN	N	[illegible]	[illegible]	[illegible]	271	GLN	CA	[illegible]	[illegible]	[illegible]
271	GLN	C	[illegible]	[illegible]	[illegible]	271	GLN	O	[illegible]	[illegible]	[illegible]
271	GLN	CB	[illegible]	[illegible]	[illegible]	271	GLN	CG	[illegible]	[illegible]	[illegible]
271	GLN	CD	[illegible]	[illegible]	[illegible]	271	GLN	OE1	[illegible]	[illegible]	[illegible]
271	GLN	NE2	[illegible]	[illegible]	[illegible]	272	ALA	N	[illegible]	[illegible]	[illegible]
272	ALA	CA	[illegible]	[illegible]	[illegible]	272	ALA	C	[illegible]	[illegible]	[illegible]
272	ALA	O	[illegible]	[illegible]	[illegible]	272	ALA	CB	[illegible]	[illegible]	[illegible]
273	ALA	N	[illegible]	[illegible]	[illegible]	273	ALA	CA	[illegible]	[illegible]	[illegible]
273	ALA	C	[illegible]	[illegible]	[illegible]	273	ALA	O	[illegible]	[illegible]	[illegible]
273	ALA	CB	[illegible]	[illegible]	[illegible]	274	ALA	N	[illegible]	[illegible]	[illegible]
274	ALA	CB	[illegible]	[illegible]	[illegible]	274	ALA	CA	[illegible]	[illegible]	[illegible]
274	ALA	C	[illegible]	[illegible]	[illegible]	274	ALA	O	[illegible]	[illegible]	[illegible]
275	GLN	N	[illegible]	[illegible]	[illegible]	275	GLN	CA	[illegible]	[illegible]	[illegible]
275	GLN	C	[illegible]	[illegible]	[illegible]	275	GLN	O	[illegible]	[illegible]	[illegible]
275	GLN	[illegible]	[illegible]	[illegible]	[illegible]	275	GLN	CB	[illegible]	[illegible]	[illegible]
275	GLN	CG	[illegible]	[illegible]	[illegible]	275	GLN	CD	[illegible]	[illegible]	[illegible]
275	GLN	OE1	[illegible]	[illegible]	[illegible]	275	GLN	NE2	[illegible]	[illegible]	[illegible]

The above structural studies together with the kinetic data presented herein and elsewhere (Philipp, M., et al. (1983) Mol. Cell. Biochem. 51, 5-32; Svendsen, I.B. (1976) Carlsberg Res. Comm. 41, 237-291; Markland, S.F. Id; Stauffe, D.C., et al. (1965) J. Biol. Chem. 244, 5333-5338) indicate that the subsites in the binding cleft of subtilisin are capable of interacting with substrate amino acid residues from P-4 to P-2'.

The most extensively studied of the above residues are Gly166, Gly169 and Ala152. These amino acids were identified as residues within the S-1 subsite. As seen in Fig. 3, which is a stereoview of the S-1 subsite, Gly166 and Gly169 occupy positions at the bottom of the S-1 subsite, whereas Ala152 occupies a position near the top of S-1, close to the catalytic Ser221.

All 19 amino acid substitutions of Gly166 and Gly169 have been made. As will be indicated in the examples which follow, the preferred replacement amino acids for Gly166 and/or Gly169 will depend on the specific amino acid occupying the P-1 position of a given substrate.

The only substitutions of Ala152 presently made and analyzed comprise the replacement of Ala152 with Gly and Ser. The results of these substitutions on P-1 specificity will be presented in the examples.

In addition to those residues specifically associated with specificity for the P-1 substrate amino acid, Tyr104 has been identified as being involved with P-4 specificity. Substitutions at Phe189 and Tyr217, however, are expected to respectively effect P-2' and P-1' specificity.

The catalytic activity of subtilisin has also been modified by single amino acid substitutions at Asn155. The catalytic triad of subtilisin is shown in Fig. 4. As can be seen, Ser221, His64 and Asp32 arc positioned to facilitate nucleophilic attach by the serine hydoxylate on the carbonyl of the scissile peptide bond. Crystallographic studies of subtilisin (Robertus, et al. (1972) Biochem. 11, 4293-4303; Matthews, et al. (1975) J. Biol. Chem. 250, 7120-7126; Poulos, et al. (1976) J. Biol. Chem. 250, 1097-1103) show that two hydrogen bonds are formed with the oxyanion of the substrate transition state. One hydrogen bond donor is from the catalytic serine-221 main-chain amide while the other is from one of the NE2 protons of the asparagine-155 side chain. See Fig. 4.

Asn155 was substituted with Ala, Asp, His, Glu and Thr. These substitutions were made to investigate the the stabilization of the charged tetrahedral intermediate of the transition state complex by the potential hydrogen bond between the side chain of Asn155 and the oxyanion of the intermediate. These particular substitutions caused large decreases in substrate turnover, kcat (200 to 4,000 fold), marginal decreases in substrate binding Km (up to 7 fold), and a loss in transition state stabilization energy of 2.2 to 4.7 kcal/mol. The retention of Km and the drop in kcat will make these mutant enzymes useful as binding proteins for specific; peptide sequences, the nature of which will be determined by the specificity of the precursor protease.

Various other amino acid residues have been identified which affect alkaline stability. In some cases, mutants having altered alkaline stability also have altered thermal stability.

In B amyloliquefaciens subtilisin residues Asp36, Ile107, Lys170, Ser204 and Lys213 have been identified as residues which upon substitution with a different amino acid alter the alkaline stability of the mutated enzyme as compared to the precursor enzyme. The substitution of Asp36 with Ala and the substitution of Lys170 with Glu each resulted in a mutant enzyme having a lower alkaline stability as compared to the wild type subtilisin. When Ile107 was substituted with Val, Ser204 substituted with Cys, Arg or Leu or Lys213 substituted with Arg, the mutant subtilisin had a greater alkaline stability as compared

to the wild type subtilisin. However, the mutant Ser204P demonstrated a decrease in alkaline stability.

In addition, other residues, identified as being associated with the modification of other properties of subtilisin, also affect alkaline stability. These residues include Ser24, Met50, Glu156, Gly166, Gly169 and Tyr217. Specifically the following particular substitutions result in an increased alkaline stability: Ser24C, Met50F, Gly156Q or S, Gly166A, H, K, N or Q, Gly169S or A, and Tyr217F, K, R or L. The mutant Met50V, on the other hand, results in a decrease in the alkaline stability of the mutant subtilisin as compared to wild type subtilisin.

Other residues involved in alkaline stability based on the alkaline stability screen include Asp197 and Met222. Particular mutants include Asp197(R or A) and Met 222 (all other amino acids).

Various other residues have been identified as being involved in thermal stability as determined by the thermal stability screen herein. These residues include the above identified residues which effect alkaline stability and Met199 and Tyr21. These latter two residues are also believed to be important for alkaline stability. Mutants at these residues include I199 and F21.

The amino acid sequence of B. amyloliquefaciens substilisin has also been modified by substituting two or more amino acids of the wild-type sequence. Six categories of multiply substituted mutant subtilisin have been identified. The first two categories comprise thermally and oxidatively stable mutants. The next three other categories comprise mutants which combine the useful properties of any of several single mutations of B. amyloliquefaciens subtilisin. The last category comprises mutants which have modified alkaline and/or thermal stability.

The first category comprises double mutants in which two cysteine residues have been substituted at various amino acid residue positions within the subtilisin molecule. Formation of disulfide bridges between the two substituted cysteine residues results in mutant subtilisins with altered thermal stability and catalytic activity. These mutants include A21/C22/C87 and C24/C87 which will be described in more detail in Example 11.

The second category of multiple subtilisin mutants comprises mutants which are stable in the presence of various oxidizing agents such as hydrogen peroxide or peracids. Examples 1 and 2 describe these mutants which include F50/I124/Q222, F50/I124, F50/Q222, F50/L124/Q222, I124/Q222 and L124/Q222.

The third category of multiple subtilisin mutants comprises mutants with substitutions at position 222 combined with various substitutions at positions 166 or 169. These mutants, for example, combine the property of oxidative stability of the A222 mutation with the altered substrate specificity of the various 166 or 169 substitutions. Such multiple mutants include A166/A222, A166/C222, F166/C222, K166/A222, K166/C222, V166/A222 and V166/C222. The K166/A222 mutant subtilisin, for example, has a kcat/Km ratio which is approximately two times greater than that of the single A222 mutant subtilisin when compared using a substrate with phenylalanine as the P-1 amino acid. This category of multiple mutant is described in more detail in Example 12.

The fourth category of multiple mutants combines substitutions at position 156 (Glu to Q or S) with the substitution of Lys at position 166. Either of these single mutations improve enzyme performance upon substrates with glutamate as the P-1 amino acid. When these single mutations are combined, the resulting multiple enzyme mutants perform better than either precursor. See Example 9.

The fifth category of multiple mutants contain the substitution of up to four amino acids of the B. amyloliquefaciens subtilisin sequence. These mutants have specific properties which are virtually identicle to the properties of the subtilisin from B. licheniformis. The subtilisin from B. licheniformis differs from B. amyloliquefaciens subtilisin at 87 out of 275 amino acids. The multiple mutant F50/S156/A169/L217 was found to have similar substrate specificity and kinetics to the licheniformis enzyme. (See Example 13.) However, this is probably due to only three of the mutations (S156, A169 and L217) which are present in the substrate binding region of the enzyme. It is quite surprising that, by making only three changes out of the 87 different amino acids between the sequence of the two enzymes, the B. amyloliquifaciens enzyme was converted into an enzyme with properties similar to B. licheniformis enzyme. Other enzymes in this series include F50/Q156/N166/L217 and F50/S156/L217.

The sixth category of multiple mutants includes the combination of substitutions at position 107 (Ile to V) with the substitution of Lys at position 213 with Arg, and the combination of substitutions of position 204 (preferably Ser to C or L but also to all other amino acids) with the substituion of Lys at position 213 with R. Other multiple mutants which have altered alkaline stability include Q156/K166, Q156/N166, S156/K166, S156/N166 (previously identified as having altered substrate specificity), and F50/S156/A169/L217 (previously identified as a mutant of B. amyloliquifaciens subtilisin having properties similar to subtilisin from B. licheniformis). The mutant F50/V107/R213 was constructed based on the observed increase in alkaline stability for the single mutants F50, V107 and R213. It was determined that the V107/R213 mutant had an increased alkaline stability as compared to the wild type subtilisin. In this particular mutant, the increased

alkaline stability was the result of the cumulative stability of each of the individual mutations. Similarly, the mutant F50/V107/R213 had an even greater alkaline stability as compared to the V107/R213 mutant indicating that the increase in the alkaline stability due to the F50 mutation was also cumulative.

Table IV summarizes the multiple mutants which have been made including those not mentioned above. In addition, based in part on the above results, substitution at the following residues in subtilisin is expected to produce a multiple mutant having increased thermal and alkaline stability: Ser24, Met50, Ile107, Glu156, Gly166, Gly169, Ser204, Lys213, Gly215, and Tyr217.

TABLE IV

Double Mutants	Triple, Quadruple or Other Multiple
C22/C87	F50/I124/Q222
C24/C87	F50/L124/Q222
V45/V48	F50/L124/A222
C49/C94	A21/C22/C87
C49/C95	F50/S156/N166/L217
C50/C95	F50/Q156/N166/L217
C50/C110	F50/S156/A169/L217
F50/I124	F50/S156/L217
F50/Q222	F50/Q156/K166/L217
I124/Q222	F50/S156/K166/L217
Q156/D166	F50/Q156/K166/K217
Q156/K166	F50/S156/K166/K217
Q156/N166	F50/V107/R213
S156/D166	[S153/S156/A158/G159/S160/Δ161-164/I165/S166/A169/R170]
S156/K166	
S156/N166	L204/R213
S156/A169	R213/204A, E, Q, D, N, G, K, V, R, T, P, I, M, F, Y, W or H
A166/A222	
A166/C222	
F166/A222	V107/R213
F166/C222	
K166/A222	
K166/C222	
V166/A222	
V166/C222	
A169/A222	
A169/A222	
A169/C222	
A21/C22	

In addition to the above identified amino acid residues, other amino acid residues of subtilisin are also considered to be important with regard to substrate specificity. Mutation of each of these residues is expected to produce changes in the substrate specificity of subtilisin. Moreover, multiple mutations among these residues and among the previously identified residues are also expected to produce subtilisin mutants having novel substrate specificity.

Particularly important residues are His67, Ile107, Leu126 and Leu135. Mutation of His67 should alter the S-1' subsite, thereby altering the specificity of the mutant for the P-1' substrate residue. Changes at this position could also affect the pH activity profile of the mutant. This residue was identified based on the inventor's substrate modeling from product inhibitor complexes.

Ile107 is involved in P-4 binding. Mutation at this position thus should alter specificity for the P-4 substrate residue in addition to the observed effect on alkaline stability. Ile107 was also identified by molecular modeling from product inhibitor complexes.

The S-2 binding site includes the Leu126 residue. Modification at this position should therefore affect P-2 specificity. Moreover, this residue is believed to be important to convert subtilisin to an amino peptidase.

The pH activity profile should also be modified by appropriate substitution. These residues were identified from inspection of the refined model, the three dimensional structure from modeling studies. A longer side chain is expected to preclude binding of any side chain at the S-2 subsite. Therefore, binding would be restricted to subsites S-1, S-1', S-2', S-3' and cleavage would be forced to occur after the amino terminal peptide.

Leu135 is in the S-4 subsite and if mutated should alter substrate specificity for P-4 if mutated. This residue was identified by inspection of the three-dimensional structure and modeling based on the product inhibitor complex of F222.

In addition to theses sites, specific amino acid residues within the segments 97-103, 126-129 and 213-215 are also believed to be important to substrate binding.

Segments 97-103 and 126-129 form an antiparallel beta sheet with the main chain of substrate residues P-4 through P-2. Mutating residues in those regions should affect the substrate orientation through main chain (enzyme) - main chain (substrate) interactions, since the main chain of these substrate residues do not interact with these particular residues within the S-4 through S-2 subsites.

Within the segment 97-103, Gly97 and Asp99 may be mutated to alter the position of residues 101-103 within the segment. Changes at these sites must be compatible, however. In B. amyloliquifaciens subtilisin Asp99 stabilizes a turn in the main chain tertiary folding that affects the direction of residues 101-103. B. licheniformis subtilisin Asp97, functions in an analogous manner.

In addition to Gly97 and Asp99, Ser101 interacts with Asp99 in B. amyliquefaciens subtilisin to stabilize the same main chain turn. Alterations at this residue should alter the 101-103 main chain direction. Mutations at Glu103 are also expected to affect the 101-103 main chain direction.

The side chain of Gly102 interacts with the substrate P-3 amino acid. Side chains of substituted amino acids thus are expected to significantly affect specificity for the P-3 substrate amino acids.

All the amino acids within the 127-129 segment are considered important to substrate specificity. Gly127 is positioned such that its side chain interacts with the S-1 and S-3 subsites. Altering this residue thus should alter the specificity for P-1 and P-3 residues of the substrate.

The side chain of Gly128 comprises a part of both the S-2 and S-4 subsites. Altered specificity for P-2 and P-4 therefore would be expected upon mutation. Moreover, such mutation may convert subtilisin into an amino peptidase for the same reasons substitutions of Leu126 would be expected to produce that result.

The Pro129 residue is likely to restrict the conformational freedom of the sequence 126-133, residues which may play a major role in determining P-1 specificity. Replacing Pro may introduce more flexibility thereby broadening the range of binding capabilities of such mutants.

The side chain of Lys213 is located within the S-3 subsite. All of the amino acids within the 213-215 segment are also considered to be important to substrate specificity. Accordingly, altered P-3 substrate specificity is expected upon mutation of this residue.

The Tyr214 residue does not interact with substrate but is positioned such that it could affect the conformation of the hair pin loop 204-217.

Finally, mutation of the Gly215 residue should affect the S-3' subsite, and thereby alter P-3' specificity.

In addition to the above substitutions of amino acids, the insertion or deletion of one or more amino acids within the external loop comprising residues 152-172 may also affect specificity. This is because these residues may play a role in the "secondary contact region" described in the model of streptomyces subtilisin inhibitor complexed with subtilisin. Hirono, et al. (1984) J. Mol. Biol. 178, 389-413. Thermitase K has a deletion in this region, which eliminates several of these "secondary contact" residues. In particular, deletion of residues 161 through 164 is expected to produce a mutant subtilisin having modified substrate specificity. In addition, a rearrangement in this area induced by the deletion should alter the position of many residues involved in substrate binding, predominantly at P-1. This, in turn, should affect overall activity against proteinaceous substrates

The effect of deletion of residues 161 through 164 has been shown by comparing the activity of the wild type (WT) enzyme with a mutant enzyme containing this deletion as well as multiple substitutions (i.e., S153/S156/A158/G159/S160/Δ161-164/I165/S166/A169/R170). This produced the following results:

TABLE V

	kcat	Km	kcat/Km
WT	50	1.4×10^{-4}	3.6×10^{5}
Deletion mutant	8	5.0×10^{-6}	1.6×10^{6}

The WT has a kcat 6 times greater than the deletion mutant but substrate binding is 28 fold tighter by the deletion mutant. The overall efficiency of the deletion mutant is thus 4.4 times higher than the WT enzyme.

All of these above identified residues which have yet to be substituted, deleted or inserted into are presented in Table VI.

TABLE VI

Substitution/Insertion/Deletion Residues	
His67	Ala152
Leu126	Ala153
Leu135	Gly154
Gly97	Asn155
Asp99	Gly156
Ser101	Gly157
Gly102	Gly160
Glu103	Thr158
Leu126	Ser159
Gly127	Ser161
Gly128	Ser162
Pro129	Ser163
Tyr214	Thr164
Gly215	Val165
Gly166	Gly169
Tyr167	Lys170
Pro168	Tyr171
	Pro172

The following disclosure is intended to serve as a representation of embodiments herein, and should not be construed as limiting the scope of this application. These specific examples disclose the construction of certain of the above identified mutants. The construction of the other mutants, however, is apparent from the disclosure herein and that presented in EPO Publication No. 0130756.

All literature citations are expressly incorporated by reference.

EXAMPLE 1

Identification of Peracid Oxidizable Residues of Subtilisin Q222 and L222

As shown in Figures 6A and 6B, organic peracid oxidants inactivate the mutant subtilisins Met222L and Met222Q (L222 and Q222). This example describes the identification of peracid oxidizable sites in these mutant subtilisins.

First, the type of amino acid involved in peracid oxidation was determined. Except under drastic conditions (Means, G.E., et al. (1971) Chemical Modifications of Proteins, Holden-Day, S.F., CA, pp. 160-162), organic peracids modify only methionine and tryptophan in subtilisin. Difference spectra of the enzyme over the 250nm to 350nm range were determined during an inactivation titration employing the reagent, diperdodecanoic acid (DPDA) as oxidant. Despite quantitative inactivation of the enzyme, no change in absorbance over this wavelength range was noted as shown in Figures 7A and 7B indicating that tryptophan was not oxidized. Fontana, A., et al. (1980) Methods in Peptide and Protein Sequence Analysis - (C. Birr ed.) Elsevier, New York, p. 309. The absence of tryptophan modification implied oxidation of one or more of the remaining methionines of B. amyloliquefaciens subtilisin. See Figure 1.

To confirm this result the recombinant subtilisin Met222F was cleaved with cyanogen bromide (CNBr) both before and after oxidation by DPDA. The peptides produced by CNBr cleavage were analyzed on high resolution SDS-pyridine peptide gels (SPG).

Subtilisin Met222F (F222) was oxidized in the following manner. Purified F222 was resuspended in 0.1 M sodium borate pH 9.5 at 10 mg/ml and was added to a final concentration of 26 diperdodecanoic acid

(DPDA) at 26 mg/ml was added to produce an effective active oxygen concentration of 30 ppm. The sample was incubated for at least 30 minutes at room temperature and then quenched with 0.1 volume of 1 M Tris pH 8.6 buffer to produce a final concentration of 0.1 M Tris pH 8.6). 3mM phenylmethylsulfonyl fluoride (PMSF) was added and 2.5 ml of the sample was applied to a Pharmacia PD10 column equilibrated in 10 mM sodium phosphate pH 6.2, 1 mM PMSF. 3.5 ml of 10 mM sodium phosphate pH6.2, 1mM PMSF was applied and the eluant collected.

F222 and DPDA oxidized F222 were precipitated with 9 volumes of acetone at -20°C. The samples were resuspended at 10 mg/ml in 8M urea in 88% formic acid and allowed to sit for 5 minutes. An equal volume of 200 mg/ml CNBr in 88% formic acid was added (5 mg/ml protein) and the samples incubated for 2 hours at room temperature in the dark. Prior to gel electrophoresis, the samples were lyophilized and resuspended at 2-5 mg/ml in sample buffer (1% pyridine, 5% $NaDodSO_4$, 5% glycerol and bromophenol blue) and disassociated at 95°C for 3 minutes.

The samples were electrophoresed on discontinuous polyacrylamide gels (Kyte, J., et al. (1953) Anal. Bioch. 133, 515-522). The gels were stained using the Pharmacia silver staining technique (Sammons, D.W., et al. (1981) Electrophoresis 2 135-141).

The results of this experiment are shown in Figure 8. As can be seen, F222 treated with CNBr only gives nine resolved bands on SPG. However, when F222 is also treated with DPDA prior to cleavage, bands X, 7 and 9 disappear whereas bands 5 and 6 are greatly increased in intensity.

In order to determine which of the methionines were effected, each of the CNBr peptides was isolated by reversed phase HPLC and further characterized. The buffer system in both Solvent A (aqueous) and Solvent B (organic) for all HPLC separations was 0.05% triethylamime/trifloroacetic acid (TEA-TFA). In all cases unless noted, solvent A consisted of 0.05% TEA-TFA in H_20, solvent B was 0.05% TEA-TFA in 1-propanol, and the flow rate was 0.5 ml/minute.

For HPLC analysis, two injections of 1 mg enzyme digest were used. Three samples were acetone precipitated, washed and dried. The dried 1 mg samples were resuspended at 10 mg/ml in 8M urea, 88% formic acid; an equal volume of 200 mg/ml CNBr in 88% formic acid was added (5 mg/ml protein). After incubation for 2 hours in the dark at room temperature, the samples were desalted on a 0.8 cm X 7 cm column of Tris Acryl GF05 coarse resin (IBF, Paris, France) equilibrated with 40% solvent B, 60% solvent A. 200 ul samples were applied at a flow rate of 1 ml a minute and 1.0-1.2 ml collected by monitoring the absorbance at 280nm. Prior to injection on the HPLC, each desalted sample was diluted with 3 volumes of solvent A. The samples were injected at 1.0 ml/min (2 minutes) and the flow then adjusted to 0.5 ml/min (100% A). After 2 minutes, a linear gradient to 60% B at 1.0% B/min was initiated. From each 1 mg run, the pooled peaks were sampled (50ul) and analyzed by gel electrophoresis as described above.

Each polypeptide isolated by reversed phase HPLC was further analyzed for homogeneity by SPG. The position of each peptide on the known gene sequence (Wells, J.A., et al. (1983) Nucleic Acids Res. 11 7911-7924) was obtained through a combination of amino acid compositional analysis and, where needed, amino terminal sequencing.

Prior to such analysis the following peptides were to rechromatographed.

1. CNBr peptides from F222 not treated with DPDA:

Peptide 5 was subjected to two additional reversed phase separations. The 10 cm C4 column was equilibrated to 80%A/ 20%B and the pooled sample applied and washed for 2 minutes. Next an 0.5% ml B/min gradient was initiated. Fractions from this separation were again rerun, this time on the 25 cm C4 column, and employing 0.05% TEA-TFA in acetonitrile/1-propanol (1:1) for solvent B. The gradient was identical to the one just described.

Peptide "X" was subjected to one additional separation after the initial chromatography. The sample was applied and washed for 2 minutes at 0.5ml/min (100%A), and a 0.5% ml B/min gradient was initiated.

Peptides 7 and 9 were rechromatographed in a similar manner to the first rerun of peptide 5.

Peptide 8 was purified to homogeneity after the initial separation.

2. CNBr Peptides from DPDA Oxidized F222:

Peptides 5 and 6 from a CNBr digest of the oxidized F222 were purified in the same manner as peptide 5 from the untreated enzyme.

Amino acid compositional analysis was obtained as follows. Samples (-1nM each amino acid) were dried, hydrolyzed in vacuo with 100 ul 6N HCl at 106°C for 24 hours and then dried in a Speed Vac. The samples were analyzed on a Beckmann 6300 AA analyzer employing ninhydrin detection.

Amino terminal sequence data was obtained as previously described (Rodriguez, H., et al. (1984) Anal. Biochem. 134, 538-547).

The results are shown in Table VII and Figure 9.

TABLE VII

Amino and COOH terminii of CNBr fragments Terminus and Method		
Fragment	amino, method	COOH, method
X	1, sequence	50, composition
9	51, sequence	119, composition
7	125, sequence	199, composition
8	200, sequence	275, composition
5ox	1, sequence	119, composition
6ox	120, composition	199, composition

Peptides 5ox and 6ox refer to peptides 5 and 6 isolated from CNBr digests of the oxidized protein where their respective levels are enhanced.

From the data in Table VII and the comparison of SPG tracks for the oxidized and native protein digests in Figure 8, it is apparent that (1) Met50 is oxidized leading to the loss of peptides X and 9 and the appearance of 5; and (2) Met124 is also oxidized leading to the loss of peptide 7 and the accumulation of peptide 6. Thus oxidation of B. amyloliquifaciens subtilisin with the peracid, diperdococanoic acid leads to the specific oxidation of methionine at residues 50 and 124.

EXAMPLE 2

Substitution at Met50 and Met124 in Subtilisin Met222Q

The choice of amino acid for substitution at Met50 was based on the available sequence data for subtilisins from B. licheniformis (Smith, E.C., et al. (1968) J. Biol. Chem. 243, 2184-2191), B.DY (Nedkov, P., et al. (1983) Hoppe Sayler's Z. Physiol. Chem. 364 1537-1540), B. amylosacchariticus (Markland, F.S., et al. (1967) J. Biol. Chem. 242 5198-5211) and B. subtilis (Stahl, M.L., et al. (1984) J. Bacteriol. 158, 411-418). In all cases, position 50 is a phenylalanine. See Figure 5. Therefore, Phe50 was chosen for construction.

At position 124, all known subtilisins possess a methionine. See Figure 5. Molecular modelling of the x-ray derived protein structure was therefore rehired to determine the most probable candidates for substitution. From all 19 candidates, isoleucine and leucine were chosen as the best residues to employ. In order to test whether or not modification at one site but not both was sufficient to increase oxidative stability, all possible combinations were built on the Q222 backbone (F50/Q222, I124/Q222, F50/I124/Q222).

A. Construction of Mutations Between Codons 45 and 50

All manipulations for cassette mutagenesis were carried out on pS4.5 using methods disclosed in EPO Publication No. 0130756 and Wells, J.A., et al, (1985) Gene 34, 315-323. The pΔ50 in Fig. 10, line 4, mutations was produced using the mutagenesis primer shown in Fig. 10, line 6, and employed an approach designated as restriction-purification which is described below. Briefly, a M13 template containing the subtilisin gene, M13mp11-SUBT was used for heteroduplex synthesis (Adelman, et al (1983), DNA 2, 183-193). Following transfection of JM101 (ATCC 33876), the 1.5 kb EcoRI-BamHI fragment containing the subtilisin gene was subcloned from M13mp11 SUBT rf into a recipient vector fragment of pBS42 the construction of which is described in EPO Publication No. 0130756. To enrich for the mutant sequence (pΔ50, line 4), the resulting plasmid pool was digested with KpnI, and linear molecules were purified by polyacrylamide gel electrophoresis. Linear molecules were ligated back to a circular form, and transformed into E. coli MM294 cells (ATCC 31446). Isolated plasmids were screened by restriction analysis for the KpnI, site. KpnI$^+$ plasmids were sequenced and confirmed the pΔ50 sequence. Asterisks in Figure 11 indicate the bases that are mutated from the wid type sequence (line 4). pΔ50 (line 4) was cut with StuI and EcoRI and the 0.5 Kb fragment containing the 5' half of the subtilisin gene was purified (fragment 1). pΔ50 (line 4) was digested with KpnI and EcoRI and the 4.0 Kb fragment containing the 3' half of the subtilisin gene and vector sequences was purified (fragment 2). Fragments 1 and 2 (line 5), and duplex DNA

cassettes coding for mutations desired (shaded sequence, line 6) were mixed in a molar ratio of 1:1:10, respectively. For the particular construction of this example the DNA cassette contained the triplet TTT for codon 50 which encodes Phe. This plasmid was designated pF50. The mutant subtilisin was designated F50.

B. Construction of Mutation Between Codons 122 and 127

The procedure of Example 2A was followed in substantial detail except that the mutagenesis primer of Figure 11, line 7 was used and restriction-purification for the EcoRV site in pΔ124 was used. In addition, the DNA cassette (shaded sequence, Figure 11, line 6) contained the triplet ATT for codon 124 which encodes Ile and CTT for Leu. Those plasmids which contained the substitution of Ile for Met124were designeated pI124. The mutant subtilisin was designated I124.

C. Construction of Various F50/I124/Q222 Multiple Mutants

The triple mutant, F50/I124/Q222, was constructed from a three-way ligation in which each fragment contained one of the three mutations. The single mutant Q222 (pQ222) was prepared by cassette mutagenesis as described in EPO Publication No. 0130756. The F50 mutation was contained on a 2.2kb AvaII to PvuII fragment from pF50; the I124 mutation was contained on a 260 bp PvuII to AvaII fragment from pI124; and the Q222 mutation was contained on 2.7 kb AvaII to AvaII fragment from pQ222. The three fragments were ligated together and transformed into E. coli MM294 cells. Restriction analysis of plasmids from isolated transformants confirmed the construction. To analyze the final construction it was convenient that the AvaII site at position 798 in the wild-type subtilisin gene was eliminated by the I124 construction.

The F50/Q222 and I124/Q222 mutants were constructed in a similar manner except that the appropriate fragment from pS4.5 was used for the final construction.

D. Oxidative Stability of Q222 Mutants

The above mutants were analyzed for stability to peracid oxidation. As shown in Fig. 12, upon incubation with diperdodecanoic acid (protein 2mg/mL, oxidant 75ppm[0]), both the I124/Q222 and the F50/I124/Q222 are completely stable whereas the F50/Q222 and the Q222 are inactivated. This indicates that conversion of Met124 to I124 in subtilisin Q222 is sufficient to confer resistance to organic peracid oxidants.

EXAMPLE 3

Subtilisin Mutants Having Altered Substrate Specificity-Hydrophobic Substitutions at Residues 166

Subtilisin contains an extended binding cleft which is hydrophobic in character. A conserved glycine at residue 166 was replaced with twelve non-ionic amino acids which can project their side-chains into the S-1 subsite. These mutants were constructed to determine the effect of changes in size and hydrophobicity on the binding of various substrates.

A. Kinetics for Hydrolysis of Substrates Having Altered P-1 Amino Acids by Subtilisin from B. Amyloliquefaciens

Wild-type subtilisin was purified from B. subtilis culture supernatants expressing the B. amyloliquefaciens subtilisin gene (Wells, J.A., et al. (1983) Nucleic Acids Res. 11, 7911-7925) as previously described (Estell, D.A., et al. (1985) J. Biol. Chem. 260, 6518-6521). Details of the synthesis of tetrapeptide substrates having the form succinyl-L-AlaL-AlaL-ProL-[X]-p-nitroanilide (where X is the P1 amino acid) are described by DelMar, E.G., et al. (1979) Anal. Biochem. 99, 316-320. Kinetic parameters, Km(M) and kcat-(s^{-1}) were measured using a modified progress curve analysis (Estell, D.A., et al. (1985) J. Biol. Chem. 260, 6518-6521). Briefly, plots of rate versus product concentration were fit to the differential form of the rate equation using a non-linear regression algorithm. Errors in kcat and Km for all values reported are less than five percent. The various substrates in Table VIII are ranged in order of decreasing hydrophobicity. Nozaki, Y. (1971), J. Biol. Chem. 246, 2211-2217; Tanford C. (1978) Science 200, 1012).

TABLE VIII

P1 substrate Amino Acid	kcat(S^{-1})	1/Km(M^{-1})	kcat/Km (s^{-1}M-1)
Phe	50	7,100	360,000
Tyr	28	40,000	1,100,000
Leu	24	3,100	75,000
Met	13	9,400	120,000
His	7.9	1,600	13,000
Ala	1.9	5,500	11,000
Gly	0.003	8,300	21
Gln	3.2	2,200	7,100
Ser	2.8	1,500	4,200
Glu	0.54	32	16

The ratio of kcat/Km (also referred to as catalytic efficienty) is the apparent second order rate constant for the conversion of free enzyme plus substrate (E+S) to enzyme plus products (E+P) (Jencks, W.P., Catalysis in Chemistry and Enzymology (McGraw-Hill, 1969) pp. 321-436; Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287). The log (kcat/Km) is proportional to transition state binding energy, $\Delta G_T^{\ddagger}$. A plot of the log kcat/Km versus the hydrophobicity of the P1 side-chain (Figure 14) shows a strong correlation (r = 0.98), with the exception of the glycine substrate which shows evidence for non-productive binding. These data show that relative differences between transition-state binding energies can be accounted for by differences in P-1 side-chain hydrophobicity. When the transition-state binding energies are calculated for these substrates and plotted versus their respective side-chain hydrophobicities, the line slope is 1.2 (not shown). A slope greater than unity, as is also the case for chymotrypsin (Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287; Harper, J.W., et al. (1984) Biochemistry, 23, 2995-3002), suggests that the P1 binding cleft is more hydrophobic than ethanol or dioxane solvents that were used to empirically determine the hydrophobicity of amino acids (Nozaki, Y., et al. J. Biol. Chem. (1971) 246, 2211-2217; Tanford, C. (1978) Science 200, 1012).

For amide hydrolysis by subtilisin, kcat can be interpreted as the acylation rate constant and Km as the dissociation constant, for the Michaelis complex (E•S), Ks. Gutfreund, H., et al (1956) Biochem. J. 63, 656. The fact that the log kcat, as well as log 1/Km, correlates with substrate hydrophobicity is consistent with proposals (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449; Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303) that during the acylation step the P-1 side-chain moves deeper into the hydrophobic cleft as the substrate advances from the Michaelis complex (E•S) to the tetrahedral transition-state complex (E•S‡). However, these data can also be interpreted as the hydrophobicity of the P1 side-chain effecting the orientation, and thus the susceptibility of the scissile peptide bond to nucleophilic attack by the hydroxyl group of the catalytic Ser221.

The dependence of kcat/Km on P-1 side chain hydrophobicity suggested that the kcat/Km for hydrophobic substrates may be increased by increasing the hydrophobicity of the S-1 binding subsite. To test this hypothesis, hydrophobic amino acid substitutions of Gly166 were produced.

Since hydrophobicity of aliphatic side-chains is directly proportional to side-chain surface area (Rose, G.D., et al. (1985) Science 229, 834-838; Reynolds, J.A., et al. (1974) Proc. Natl. Acad. Sci. USA 71, 2825-2927), increasing the hydrophobicity in the S-1 subsite may also sterically hinder binding of larger substrates. Because of difficulties in predicting the relative importance of these two opposing effects, we elected to generate twelve non-charged mutations at position 166 to determine the resulting specificities against non-charged substrates of varied size and hydrophobicity.

B. Cassette Mutagenesis of the P1 Binding Cleft

The preparation of mutant subtilisims containing the substitution of the hydrophobic amino acids Ala, Val and Phe into residue 166 has been described in EPO Publication No. 0130756. The same method was used to produce the remaining hydrophobic mutants at residue 166. In applying this method, two unique and silent restriction sites were introduced in the subtilisin genes to closely flank the target codon 166. As can be seen in Figure 13, the wild type sequence (line 1) was altered by site-directed mutagenesis in M13 using the indicated 37mer mutagenesis primer, to introduce a 13 bp delection (dashedline) and unique SacI and XmaI sites (underlined sequences) that closely flank codon 166. The subtilisin gene fragment was subcloned back into the E. coli - B. subtilis shuttle plasmid, pBS42, giving the plasmid pΔ166 (Figure 13,

line 2). pΔ166 was cut open with SacI and XmaI, and gapped linear molecules were purified (Figure 13, line 3). Pools of synthetic oligonucleotides containing the mutation of interest were annealed to give duplex DNA cassettes that were ligated into gapped pΔ166 (underlined and overlined sequences in Figure 13, line 4). This construction restored the coding sequence except over position 166(NNN; line 4). Mutant sequences were confirmed by dideoxy sequencing. Asterisks denote sequence changes from the wild type sequence. Plasmids containing each mutant B. amyloliquefaciens subtilisin gene were expressed at roughly equivalent levels in a protease deficient strain of B. subtilis, BG2036 as previously described. EPO Publication No. 0130756; Yang, M., et al. (1984) J. Bacteriol. 160, 15-21; Estell, D.A., et al (1985) J. Biol. Chem. 260, 6518-6521.

C. Narrowing Substrate Specificity by Steric Hindrance

To probe the change in substrate specificity caused by steric alterations in the S-1 subsite, position 166 mutants were kinetically analyzed versus P1 substrates of increasing size (i.e., Ala, Met, Phe and Tyr). Ratios of kcat/Km are presented in log form in Figure 15 to allow direct comparisons of transition-state binding energies between various enzyme-substrate pairs.

According to transition state theory, the free enery difference between the free enzyme plus substrate (E + S) and the transition state complex ($E \cdot S^{*}$) can be calculated from equation (1),

$$(1) \quad \Delta G_T^{\neq} = -RT \ln kcat/Km + RT \ln kT/h$$

in which kcat is the turnover number, Km is the Michaelis constant, R is the gas constant, T is the temperature, k is Boltzmann's constant, and h is Planck's constant. Specificity differences are ezpressed quantitatively as differences between transition state binding energies (i.e., $\Delta\Delta G_t^{*}$), and can be calculated from equation (2).

$$(2) \quad \Delta\Delta G_T^{\neq} = -RT \ln (kcat/Km)_A/(kcat/Km)_B$$

A and B represent either two different substrates assayed againt the same enzyme, or two mutant enzymes assayed against the same substrate.

As can be seen from Figure 15A, as the size of the side-chain at position 166 increases the substrate preference shifts from large to small P-1 side-chains. Enlarging the side-chain at position 166 causes kcat/Km to decrease in proportion to the size of the P-1 substrate side-chain (e.g., from Gly166 (wild-type) through W166, the kcat/Km for the Tyr substrate is decreased most followed in order by the Phe, Met and Ala P-1 substrates).

Specific steric changes in the position 166 side-chain, such as he presence of a β-hydroxyl group, β- or γ-aliphatic branching, cause large decreases in kcat/Km for larger P1 substrates. Introducing a β-hydroxyl group in going from A166 (Figure 15A) to S166 (Figure 15B), causes an 8 fold and 4 fold reduction in kcat/Km for Phe and Tyr substrates, respectively, while the values for Ala and Met substrates are unchanged. Producing a β-branched structure, in going from S166 to T166, results in a drop of 14 and 4 fold in kcat/Km for Phe and Tyr, respectively. These differences are slightly magnified for V166 which is slightly larger and isosteric with T166. Enlarging the β-branched substituents from V166 to I166 causes a lowering of kcat/Km between two and six fold toward Met, Phe and Tyr substrates. Inserting a γ-branched structure, by replacing M166 (Figure 15A) with L166 (Figure 15B), produces a 5 fold and 18 fold decrease in kcat/Km for Phe and Tyr substrates, respectively. Aliphatic γ-branched appears to induce less steric hindrance toward the Phe P-1 substrate than β-branching, as evidenced by the 100 fold decrease in kcat/Km for the Phe substrate in going from L166 to I166.

Reductions in kcat/Km resulting from increases in side chain size in the S-1 subsite, or specific structural features such as β- and γ-branching, are quantitatively illustrated in Figure 16. The kcat/Km values for the position 166 mutants determined for the Ala, Met, Phe, and Tyr P-1 substrates (top panel through bottom panel, respectively), are plotted versus the position 166 side-chain volumes (Chothia, C. (1984) Ann. Rev. Biochem. 53, 537-572). Catalytic efficiency for the Ala substrate reaches a maximum for

I166, and for the Met substrate it reaches a maximum between V166 and L166. The Phe substrate shows a broad kcat/Km peak but is optimal with A166. Here, the β-branched position 166 substitutions form a line that is parallel to, but roughly 50 fold lower in kcat/Km than side-chains of similar size [i.e., C166 versus T166, L166 versus I166). The Tyr substrate is most efficiently utilized by wild type enzyme (Gly166), and there is a steady decrease as one proceeds to large position 166 side-chains. The β-branched and γ-branched substitutions form a parallel line below the other non-charged substitutions of similar molecular volume.

The optimal substitution at position 166 decreases in volume with increasing volume of the P1 substrate [i.e., I166/Ala substrate, L166/Met substrate, A166/Phe substrate, Gly166/Tyr substrate]. The combined volumes for these optimal pairs may approximate the volume for productive binding in the S-1 subsite. For the optimal pairs, Gly166/Tyr substrate, A166/Phe substrate, L166/Met substrate, V166/Met substrate, and I166/Ala substrate, the combined volumes are 266,295,313,339 and 261 A^3, respectively. Subtracting the volume of the peptide backbone from each pair (i.e., two times the volume of glycine), an average side-chain volume of $160\pm32A^3$ for productive binding can be calculated.

The effect of volume, in excess to the productive binding volume, on the drop in transition-state binding energy can be estimated from the Tyr substrate curve (bottom panel, Figure 16), because these data, and modeling studies (Figure 2), suggest that any substitution beyond glycine causes steric repulsion. A best-fit line drawn to all the data ($r = 0.87$) gives a slope indicating a loss of roughly 3 kcal/mol in transition state binding energy per $100A^3$ of excess volume. ($100A^3$ is approximately the size of a leucyl side-chain.)

D. Enhanced Catalytic Efficiency Correlates with Increasing Hydrophobicity of the Position 166 Substitution

Substantial increases in kcat/Km occur with enlargement of the position 166 side-chain, except for the Tyr P-1 substrate (Figure 16). For example, kcat/Km increases in progressing from Gly166 to I166 for the Ala substrate (net of ten-fold), from Gly166 to L166 for the Met substrate (net of ten-fold) and from Gly166 to A166 for the Phe substrate (net of two-fold). The increases in kcat/Km cannot be entirely explained by the attractive terms in the van der Waals potential energy function because of their strong distance dependence ($1/r^6$) and because of the weak nature of these attractive forces (Jencks, W.P., Catalysis in Chemistry and Enzymology (McGraw-Hill, 1969) pp. 321-436; Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287; Levitt, M. (1976) J. Mol. Biol. 104, 59-107). For example, Levitt (Levitt, M. (1976) J. Mol. Biol. 104, 59-107) has calculated that the van der Waals attraction between two methionyl residues would produce a maximal interaction energy of roughly -0.2 kcal/mol. This energy would translate to only 1.4 fold increase in kcat/Km.

The increases of catalytic efficiency caused by side-chain substitutions at position 166 are better accounted for by increases in the hydrophobicity of the S-1 subsite. The increase kcat/Km observed for the Ala and Met substrates with increasing position 166 side-chain size would be expected, because hydrophobicity is roughly proportional to side-chain surface area (Rose, G.D., et al. (1985) Science 229, 834-838; Reynolds, J.A., et al. (1974) Proc. Natl. Acad. Sci. USA 71, 2825-2927).

Another example that can be interpreted as a hydrophobic effect is seen when comparing kcat/Km for isosteric substitutions that differ in hydrophobicity such as S166 and C166 (Figure 16). Cysteine is considerably more hydrophobic than serine (-1.0 versus +0.3 kcal/mol) (Nozaki, Y., et al. (1971) J. Biol. Chem. 246, 2211-2217; Tanford, C. (1978) Science 200, 1012). The difference in hydrophobicity correlates with the observation that C166 becomes more efficient relative to Ser166 as the hydrophobicity of the substrates increases (i.e., Ala < Met < Tye < Phe). Steric hindrance cannot explain these differences because serine is considerably smaller than cysteine (99 versus $118A^3$). Paul, I.C., Chemistry of the -SH Group (ed. S. Patai, Wiley Interscience, New York, 1974) pp. 111-149.

E. Production of an Elastase-Like Specificity in Subtilisin

The I166 mutation illustrates particularly well that large changes in specificity can be produced by altering the structure and hydrophobicity of the S-1 subsite by a single mutation (Figure 17). Progressing through the small hydrophobic substrates, a maximal specificity improvement over wild type occurs for the Val substrate (16 fold in kcat/Km). As the substrate side chain size increases, these enhancements shrink to near unity (i.e., Leu and His substrates). The I166 enzyme becomes poorer against larger aromatic substrates of increasing size (e.g., I166 is over 1,000 fold worse against the Tyr substrate than is Gly166). We interpret the increase in catalytic efficiency toward the small hydrophobic substrates for I166 compared to Gly166 to the greater hydrophobicity of isoluecine (i.e., -1.8 kcal/mol versus 0). Nozaki, Y., et al. (1971) J. Biol. Chem. 246, 2211-2217; Tanford, C. (1978) Science 200, 1012. The decrease in catalytic efficiency

toward the very large substrates for I166 versus Gly166 is attributed to steric repulsion.

The specificity differences between Gly166 and I166 are similar to the specificity differences between chymotrypsin and the evolutionary relative, elastase (Harper, J.W., et al (1984) Biochemistry 23, 2995-3002). In elastase, the bulky amino acids, Thr and Val, block access to the P-1 binding site for large hydrophobic substrates that are preferred by chymotrypsin. In addition, the catalytic efficiencies toward small hydrophobic substrates are greater for elastase than for chymotrypsin as we obeseve for I166 versus Gly166 in subtilisin.

EXAMPLE 4

Substitution of Ionic Amino Acids for Gly166

The construction of subtilisin mutants containing the substitution of the ionic amino acids Asp, Asn, Gln, Lys and Ang are disclosed in EPO Publication No. 0130756. The present example describes the construction of the mutant subtilisin containing Glu at position 166 (E166) and presents substrate specificity data on these mutants. Further data on position 166 and 156 single and double mutants is presented infra.

pΔ166, described in Example 3, was digested with SacI and XmaI. The double strand DNA cassette (underlined and overlined) of line 4 in Figure 13 contained the triplet GAA for the codon 166 to encode the replacement of Glu for Gly166. This mutant plasmid designated pQ166 was propagated in BG2036 as described. This mutant subtilisin, together with the other mutants containing ionic substituent amino acids at residue 166, were isolated as described and further analyzed for variations in substrate specificity.

Each of these mutants was analyzed with the tetrapeptide substrates, succinyl-L-AlaL-AlaProL-X-p-nitroanilide, where X was Phe, Ala and Glu.

The results of this analysis are shown in Table IX.

TABLE IX

Position 166	P-1 Substrate (kcat/Km x 10^{-4})		
	Phe	Ala	Glu
Gly (wild type)	36.0	1.4	0.002
Asp (D)	0.5	0.4	<0.001
Glu (E)	3.5	0.4	<0.001
Asn (N)	18.0	1.2	0.004
Gln (Q)	57.0	2.6	0.002
Lys (K)	52.0	2.8	1.2
Arg (R)	42.0	5.0	0.08

These results indicate that charged amino acid substitutions at Gly166 have improved catalytic efficiencies (kcat/Km) for oppositely charged P-1 substrates (as much as 500 fold) and poorer catalytic efficiency for like charged P-1 substrates.

EXAMPLE 5

Substitution of Glycine at Position 169

The substitution of Gly169 in B. amyloliquefaciens subtilisin with Ala and Ser is described in EPO Publication No. 0130756. The same method was used to make the remaining 17 mutants containing all other substituent amino acids for position 169.

The construction protocol is summarized in Figure 18. The overscored and underscored double stranded DNA cassettes used contained the following triplet encoding the substitution of the indicated amino acid at residue 169.

GCT	A	ATG	M
TGT	C	AAC	N
GAT	D	CCT	P
GAA	E	CAA	Q
TTC	F	AGA	R
GGC	G	AGC	S
CAC	H	ACA	T
ATC	I	GTT	V
AAA	K	TGG	W
CTT	L	TAC	Y

Each of the plasmids containing a substituted Gly169 was designated pX169, where X represents the substituent amino acid. The mutant subtilisins were simialrly designated.

Two of the above mutant subtilisins, A169 and S169, were analyzed for substrate specificity against synthetic substrates containing Phe, Leu, Ala and Arg in the P-1 position. The following results are shown in Table X.

TABLE X

Effect of Serine and Alanine Mutations at Position 169 on P-1 Substrate Specificity				
Position 169	P-1 Substrate [kcat/Km x 10^{-4})			
	Phe	Leu	Ala	Arg
Gly (wild type)	40	10	1	0.4
A169	120	20	1	0.9
S169	50	10	1	0.6

These results indicate that substitutions of Ala and Ser at Gly169 have remarkably similar catalytic efficiencies against a range of P-1 substrates compared to their position 166 counterparts. This is probably because position 169 is at the bottom of the P-1 specificity subsite.

EXAMPLE 6

Substitution at Position 104

Tyr104 has been substituted with Ala, His, Leu, Met and Ser. The method used was a modification of the site directed mutagenesis method. According to the protocol of Figure 19, a primer (shaded in line 4) introduced a unique HindIII site and a frame shift mutation at codon 104. Restriction-purification for the unique HindIII site facilitated the isolation of the mutant sequence (line 4). Restriction-selection against this HindIII site using pimers in line 5 was used to obtain position 104 mutants.

The following triplets were used in the primers of Figure 19, line 5 for the 104 codon which substituted the following amino acids.

GCT	A	TTC	F
ATG	M	CCT	P
CTT	L	ACA	T
AGC	S	TGG	W
CAC	H	TAC	Y
CAA	Q	GTT	V
GAA	E	AGA	R
GGC	G	AAC	N
ATC	I	GAT	D
AAA	K	TGT	C

The substrates in Table XI were used to analyze the substrate specificity of these mutants. The results obtained fo H104 subtilisin are shown in Table XI.

TABLE XI

Substrate	kcat		Km		Kcat/Km	
	WT	H104	WT	H104	WT	H104
sAAPFpNA	50.0	22.0	$1.4x10^{-4}$	$7.1x10^{-4}$	$3.6x10^{5}$	$3.1x10^{4}$
sAAPApNA	3.2	2.0	$2.3x10^{-4}$	$1.9x10^{-3}$	$1.4x10^{4}$	$1x10^{3}$
sFAPFpNA	26.0	38.0	$1.8x10^{-4}$	$4.1x10^{-4}$	$1.5x10^{5}$	$9.1x10^{4}$
sFAPApNA	0.32	2.4	$7.3x10^{-5}$	$1.5x10^{-4}$	$4.4x10^{3}$	$1.6x10^{4}$

From these data it is clear that the substitution of His for Tyr at position 104 produces an enzyme which is more efficient (higher kcat/Km) when Phe is at the P-4 substrate position than when Ala is at the P-4 substrate position.

EXAMPLE 7

Substitution of Ala152

Ala152 has been substituted by Gly and Ser to determine the effect of such substitutions on substrate specificity.

The wild type DNA sequence was mutated by the V152/P153 primer (Figure 20, line 4) using the above restriction-purification approach for the new KpnI site. Other mutant primers (shaded sequences Figure 20; S152, line 5 and G152, line 6) mutated the new KpnI site away and such mutants were isolated using the restriction-selection procedure as described above for loss of the KpnI site.

The results of these substitutions for the above synthetic substrates containing the P-1 amino acids Phe, Leu and Ala are shown in Table XII.

TABLE XII

Position 152	P-1 Substrate (kcat/Km$x10^{-4}$)		
	Phe	Leu	Ala
Gly (G)	0.2	0.4	<0.04
Ala (wild type)	40.0	10.0	1.0
Ser (S)	1.0	0.5	0.2

These results indicate that, in contrast to positions 166 and 169, replacement of Ala152 with Ser or Gly causes a dramatic reduction in catalytic efficiencies across all substrates tested. This suggests Ala152, at the top of the S-1 subsite, may be the optimal amino acid because Ser end Gly ore homologous Ala substitutes.

EXAMPLE 8

Substitution at Position 156

Mutants containing the substitution of Ser and Gln for Glu156 have been constructed according to the overall method depicted in Figure 21. This method was designed to facilitate the construciton of multiple mutants at position 156 and 166 as will be described hereinafter. However, by regenerating the wild type Gly166, single mutations at Glu156 were obtained.

The plasmid pΔ166 is already depicted in line 2 of Figure 13. The synthetic oligonucleotides at the top right of Figure 21 represent the same DNA cassettes depicted in line 4 of Figure 13. The plasmid p166 in Figure 21 thus represents the mutant plasmids of Examples 3 and 4. In this particular example, p166 contains the wild type Gly166.

Construction of position 156 single mutants were prepared by ligation of the three fragments (1-3) indicated at the bottom of Figure 21. Fragment 3, containing the carboxy-terminal portion of the subtilisin gene including the wild type position 166 codon, was isolated as a 610 bp SacI-BamHI fragment. Fragment 1 contained the vector sequences, as well as the amino-terminal sequences of the subtilisin gene through codon 151. To produce fragment 1, a unique KpnI site at codon 152 was introduced into the wild type subtilisin sequence from pS4.5. Site-directed mutagenesis in M13 employed a primer having the sequence 5'-TA-GTC-GTT-GCG-GTA-CCC-GGT-AAC-GAA-3' to produce the mutation. Enrichment for the mutant sequence was accomplished by restriction with KpnI, purification and self ligation. The mutant sequence containing the KpnI site was confirmed by direct plasmid sequencing to give pV152. pV152 (~1 µg) was digested with KpnI and treated with 2 units of DNA polymerase I large fragment (Klenow fragment from Boeringer-Mannheim) plus 50 µM deoxynucleotide triphosphates at 37°C for 30 min. This created a blunt end that terminated with codon 151. The DNA was extracted with 1:1 volumes phenol and $CHCl_3$ and DNA in the aqueous phase was precipitated by addition of 0.1 volumes 5M ammonium acetate and two volumes ethanol. After centrifugation and washing the DNA pellet with 70% ethanol, the DNA was lyophilized. DNA was digested with BamHI and the 4.6kb piece (fragment 1) was purified by acrylamide gel electrophoresis followed by electroelution. Fragment 2 was a duplex synthetic DNA cassette which when ligated with fragments 1 and 3 properly restored the coding sequence except at codon 156. The top strand was synthesized to contain a glutamine codon, and the complementary bottom strand coded for serine at 156. Ligation of heterophosphorylated cassettes leads to a large and favorable bias for the phosphorylated over the non-phosphorylated oligonucleotide sequence in the final segrated plasmid product. Therefore, to obtain Q156 the top strand was phosphorylated, and annealed to the non-phosphorylated bottom strand prior to ligation. Similarly, to obtain S156 the bottom strand was phosphorylated and annealed to the non-phosphorylated top strand. Mutant sequences were isolated after ligation and transformation, and were confirmed by restriction analysis and DNA sequencing as before. To express variant subtilisins, plasmids were transformed into a subtilisin-neutral protease deletion mutant of B. subtilis, BG2036, as previously described. Cultures were fermented in shake flasks for 24 h at 37°C in LB media containing 12.5 mg/mL chloramphenicol and subtilisin was purified from culture supernatants as described. Purity of subtilisin was greater than 95% as judged by SDS PAGE.

These mutant plasmids designated pS156 and pQ156 and mutant subtilisins designated S156 and Q156 were analyzed with the above synthetic substrates where P-1 comprised the amino acids Glu, Gln, Met and Lys. The results of this analyses are presented in Example 9.

EXAMPLE 9

Multiple Mutants With Altered Substrate Specificity - Substitution at Positions 156 and 166

Single substitutions of position 166 are described in Examples 3 and 4. Example 8 describes single substitutions at position 156 as well as the protocol of Figure 21 whereby various double mutants comprising the substitution of various amino acids at positions 156 and 166 can be made. This example describes the construction and substrate specificity of subtilisin containing substitutions at position 156 and 166 and summarizes some of the data for single and double mutants at positions 156 and 166 with various substrates.

K166 is a common replacement amino acid in the 156/166 mutants described herein. The replacement of Lys for Gly166 was achieved by using the synthetic DNA cassette at the top right of Figure 21 which contained the triplet AAA for NNN. This produced fragment 2 with Lys substituting for Gly166.

The 156 substituents were Gln and Ser. The Gln and Ser substitutions at Gly156 are contained within fragment 3 (bottom right Figure 21).

The multiple mutants were produced by combining fragments 1, 2 and 3 as described in Example 8. The mutants Q156/K166 and S156/K166 were selectively generated by differential phosphorylation as described. Alternatively, the double 156/166 mutants, c.f. Q156/K166 and S156/K166, were prepared by ligation of the 4.6kb SacI-BamHI fragment from the relevant p156 plasmid containing the 0.6kb SacI-BamHI fragment from the relevant p166 plasmid.

These mutants, the single mutant K166, and the S156 and Q156 mutants of Example 8 were analyzed for substitute specificity against synthetic polypeptides containing Phe or Glu as the P-1 substrate residue. The results are presented in Table XIII.

TABLE XIII

Enzymes Compared(b)	Substrate P-1 Residue	kcat	Km	kcat/Km	kcat/Km (mutant) / kcat/Km(wt)
Glu156/Gly166 (WT)	Phe	50.00	1.4×10^{-4}	3.6×10^{5}	(1)
	Glu	0.54	3.4×10^{-2}	1.6×10^{1}	(1)
K166	Phe	20.00	4.0×10^{-5}	5.2×10^{5}	1.4
	Glu	0.70	5.6×10^{-5}	1.2×10^{4}	750
Q156/K166	Phe	30.00	1.9×10^{-5}	1.6×10^{6}	4.4
	Glu	1.60	3.1×10^{-5}	5.0×10^{4}	3100
S156/K166	Phe	30.00	1.8×10^{-5}	1.6×10^{6}	4.4
	Glu	0.60	3.9×10^{-5}	1.6×10^{4}	1000
S156	Phe	34.00	4.7×10^{-5}	7.3×10^{5}	2.0
	Glu	0.40	1.8×10^{-3}	1.1×10^{2}	6.9
E156	Phe	48.00	4.5×10^{-5}	1.1×10^{6}	3.1
	Glu	0.90	3.3×10^{-3}	2.7×10^{2}	17

As can be seen in Table XIV, either of these single mutations improve enzyme performance upon substrates with glutamate at the P-1 enzyme binding site. When these single mutations were combined, the resulting multiple enzyme mutants are better than either parent. These single or multiple mutations also alter the relative pH activity profiles of the enzymes as shown in Figure 23.

To isolate the contribution of electrostatics to substrate specificity from other chemical binding forces, these various single and double mutants were analyzed for their ability to bind and cleave synthetic substrates containing Glu, Gln, Met and Lys as the P-1 substrate amino acid. This permitted comparisons between side-chains that were more sterically similar but differed in charge (e.g., Glu versus Gln, Lys versus Met). Similarly, mutant enzymes were assayed against homologous P-1 substrates that were most sterically similar but differed in charge (Table XIV).

TABLE XIV

Kinetics of Position 156/166 Subtilisins Determined for Different P1 Substrates

Enzyme Position (a) 156	166	Net Charge (b)	P-1 Substrate log kcat/Km (log 1/Km) (c) Glu	Gln	Met	Lys
Glu	Asp	-2	n.d.	3.02 (2.56)	3.93 (2.74)	4.23 (3.00)
Glu	Glu	-2	n.d.	3.06 (2.91)	3.86 (3.28)	4.48 (3.69)
Glu	Asn	-1	1.62 (2.22)	3.85 (3.14)	4.99 (3.85)	4.15 (2.88)
Glu	Gln	-1	1.20 (2.12)	4.36 (3.64)	5.43 (4.36)	4.10 (3.15)
Gln	Asp	-1	1.30 (1.79)	3.40 (3.08)	4.94 (3.87)	4.41 (3.22)
Ser	Asp	-1	1.23 (2.13)	3.41 (3.09)	4.67 (3.68)	4.24 (3.07)
Glu	Met	-1	1.20 (2.30)	3.89 (3.19)	5.64 (4.83)	4.70 (3.89)
Glu	Ala	-1	n.d.	4.34 (3.55)	5.65 (4.46)	4.90 (3.24)
Glu	Gly(wt)	-1	1.20 (1.47)	3.85 (3.35)	5.07 (3.97)	4.60 (3.13)
Gln	Gly	0	2.42 (2.48)	4.53 (3.81)	5.77 (4.61)	3.76 (2.82)
Ser	Gly	0	2.31 (2.73)	4.09 (3.68)	5.61 (4.55)	3.46 (2.74)
Gln	Asn	0	2.04 (2.72)	4.51 (3.76)	5.79 (4.66)	3.75 (2.74)
Ser	Asn	0	1.91 (2.78)	4.57 (3.82)	5.72 (4.64)	3.68 (2.80)
Glu	Arg	0	2.91 (3.30)	4.26 (3.50)	5.32 (4.22)	3.19 (2.80)
Glu	Lys	0	4.09 (4.25)	4.70 (3.88)	6.15 (4.45)	4.23 (2.93)
Gln	Lys	+1	4.70 (4.50)	4.64 (3.68)	5.97 (4.68)	3.23 (2.75)
Ser	Lys	+1	4.21 (4.40)	4.84 (3.94)	6.16 (4.90)	3.73 (2.84)
Maximum difference: log kcat/Km (log 1/Km) (d)			3.5 (3.0)	1.8 (1.4)	2.3 (2.2)	-1.3 (-1.0)

Footnotes to Table XIV:

(a) B. subtilis, BG 2036, expressing indicated variant subtilisin were fermented and enzymes purified as previously described (Estell, et al. (1985) J. Biol. Chem. 260, 6518-6521). Wild type subtilisin is indicated (wt) containing Glu156 and Gly166.

(b) Net charge in the P-1 binding site is defined as the sum of charges from positions 156 and 166 at pH 8.6.

(c) Values for kcat(s^{-1}) and Km(M) were measured in 0.1M Tris pH 8.6 at 25°C as previously described against P-1 substrates having the form succinyl-L-AlaL-AlaL-ProL-[X]-p-nitroanilide, where X is the indicated P-1 amino acid. Values for log 1/Km are shown inside parentheses. All errors in determination of kcat/Km and 1/Km are below 5%.

(d) Because values for Glu156/Asp166(D166) are too small to determine accurately, the maximum difference taken for GluP-1 substrate is limited to a charge range of +1 to -1 charge change.

n.d. = not determined

The kcat/Km ratios shown are the second order rate constants for the conversion of substrate to product, and represent the catalytic efficiency of the enzyme. These ratios are presented in logarithmic form to scale the data, and because log kcat/Km is proportional to the lowering of transition-state activation energy (ΔG_T). Mutations at position 156 and 166 produce changes in catalytic efficiency toward Glu, Gln, Met and Lys P-1 substrates of 3100, 60, 200 and 20 fold, respectively. Making the P-1 binding-site more positively charged [e.g., compare Gln156/Lys166 (Q156/K166) versus Glu156/Met166 (Glu156/M166)] dramatically increased kcat/Km toward the Glu P-1 substrate (up to 3100 fold), and decreased the catalytic efficiency toward the Lys P-1 substrate (up to 10 fold). In addition, the results show that the catalytic efficiency of wild type enzyme can be greatly improved toward any of the four P-1 substrates by mutagenesis of the P-1 binding site.

The changes in kcat/Km ore caused predominantly by changes in 1/Km. Because 1/Km is approximately equal to 1/Ks, the enzyme-substrate association constant, the mutations primarily cause a change in substrate binding. These mutations produce smaller effects on kcat that run parallel to the effects on 1/Km. The changes in kcat suggest either an alteration in binding in the P-1 binding site in going from the Michaelis-complex E•S) to the transition-state complex (E-S≠) as previously proposed (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449; Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303), or change in the position of the scissile peptide bond over the catalytic serine in the E•S complex.

Changes in substrate preference that arise from changes in the net charge in the P-1 binding site show trends that are best accounted for by electrostatic effects (Figure 28). As the P-1 binding cleft becomes more positively charged, the average catalytic efficiency increases much more for the Glu P-1 substrate than for its neutral and isosteric P-1 homolog, Gln (Figure 28A). Furthermore, at the positive extreme both substrates have nearly identical catalytic efficiencies.

In contrast, as the P-1 site becomes more positively charged the catalytic efficiency toward the Lys P-1 substrate decreases, and diverges sharply from its neutral and isosteric homolog, Met (Figure 28B). The similar and parallel upward trend seen with increasing positive charge for the Met and Glu P-1 substrates probably results from the fact that all the substrates are succinylated on their amino-terminal end, and thus carry a formal negative charge.

The trends observed in log kcat/Km are dominated by changes in the Km term (Figures 28C and 28D). As the pocket becomes more positively charged, the log 1/Km values converge for Glu and Gln P-1 substrates (Figure 28C), and diverge for Lys and Met P-1 substrates (Figure 28D). Although less

pronounced effects are seen in log kcat, the effects of P-1 charge on log kcat parallel those seen in log 1/Km and become larger as the P-1 pocket becomes more positively charged. This may result from the fact that the transition-state is a tetrahedral anion, and a net positive charge in the enzyme may serve to provide some added stabilization to the transition-state.

The effect of the change in P-1 binding-site charge on substrate preference can be estimated from the differences in slopes between the charged and neutral isosteric P-1 substrates (Figure 28B). The average change in substrate preference (Δlog kcat/Km) between charged and neutral isosteric substrates increases roughly 10-fold as the complementary charge or the enzyme increases (Table XV). When comparing Glu versus Lys, this difference is 100-fold and the change in substrate preference appears predominantly in the Km term.

TABLE XV

Differential Effect on Binding Site Charge on log kcat/Km or (log 1/Km) for P-1 Substrates that Differ in Charge[(a)]

Change in P-1 Binding Site Charge[(b)]	Δlog kcat/Km (Δlog 1/Km)		
	GluGln	MetLys	GluLys
-2 to -1	n.d.	1.2 (1.2)	n.d.
-1 to 0	0.7 (0.6)	1.3 (0.8)	2.1 (1.4)
0 to +1	1.5 (1.3)	0.5 (0.3)	2.0 (1.5)
Avg. change in log kcat/K_m or (log 1/Km) per unit charge change	1.1 (1.0)	1.0 (0.8)	2.1 (1.5)

[(a)] The difference in the slopes of curves were taken between the P-1 substrates over the charge interval given for log (kcat/Km) (Figure 28A, B) and (log 1/Km) (Figure 28C, D). Values represent the differential effect a charge change has in distinguishing the substrates that are compared.
[(b)] Charge in P-1 binding site is defined as the sum of charges from positions 156 and 166.

The free energy of electrostatic interactions in the structure and energetics of salt-bridge formation depends on the distance between the charges and the microscopic dielectric of the media. To dissect these structural and microenvironmental effects, the energies involved in specific salt-bridges were evaluated. In addition to the possible salt-bridges shown (Figures 29A and 29B), reasonable salt-bridges can be built between a Lys P-1 substrate and Asp at position 166, and between a Glu P-1 substrate and a Lys at position 166 (not shown). Although only one of these structures is confirmed by X-ray crystalography (Poulos, T.L., et al. (1976) J. Mol. Biol. 257 1097-1103), all models have favorable torsion angles (Sielecki, A.R., et al. (1979) J. Mol. Biol. 134, 781-804), and do not introduce unfavorable van der Waals contacts.

The change in charged P-1 substrate preference brought about by formation of the model salt-bridges above are shown in Table XVI.

TABLE XVI

Effect of Salt Bridge Formation Between Enzyme and Substrate on P1 Substrate Preference[(a)]

Enzymes Compared[(b)] 1	2	Enzyme Position Changed	P-1 Substrates Compared	Substrate[(d)] Preference $\Delta\log$ (kcat/Km) 1	2	Change in Substrate Preference $\Delta\Delta\log$ (kcat/Km) (1-2)
Glu156/Asp166	Gln156/Asp166	156	LysMet	+0.30	-0.53	0.83
Glu156/Asn166	Gln156/Asn166	156	LysMet	-0.84	-2.04	1.20
Glu156/Gly166	Gln156/Gly166	156	LysMet	-0.47	-2.10	1.63
Glu156/Lsy-166	Gln156/Lys166	156	LysMet	-1.92	-2.74	0.82
				Ave $\Delta\Delta\log$ (kcat/Km)		1.10 ± 0.3
Glu156/Asp166	Glu156/Asn166	166	LysMet	+0.30	-0.84	1.14
Glu156/Glu166	Glu156/Glu166	166	LysMet	+0.62	-1.33	1.95
Gln156/Asp166	Gln156/Asn166	166	LysMet	-0.53	-2.04	1.51
Ser156/Asp166	Ser156/Asn166	166	LysMet	-0.43	-2.04	1.61
Glu156/Lys166	Glu156/Met166	166	GluGln	-0.63	-2.69	2/06
				Ave $\Delta\Delta\log$ (kcat/Km)		1.70 ± 0.3

Footnotes to Table XVI:

(a) Molecular modeling shows it is possible to form a salt bridge between the indicated charged P-1 substrate and a complementary charge in the P-1 binding site of the enzyme at the indicated position changed.

(b) Enzymes compared have sterically similar amino acid substitutions that differ in charge at the indicated position.

(c) The P-1 substrates compared are structurally similar but differ in charge. The charged P-1 substrate is complementary to the charge change at the position indicated between enzymes 1 and 2.

(d) Date from Table XIV was used to compute the difference in log (kcat/Km) between the charged and the non-charged P-1 substrate (i.e., the substrate preference). The substrate preference is shown separately for enzyme 1 and 2.

(e) The difference in substrate preference between enzyme 1 (more highly charged) and enzyme 2 (more neutral) represents the rate change accompanying the electrostatic interaction.

The difference between catalytic efficiencies (i.e., Δlog kcat/Km) for the charged and neutral P-1 substrates (e.g., Lys minus Met or Glu minus Gln) give the substrate preference for each enzyme. The change in substrate preference (ΔΔlog kcat/Km) between the charged and more neutral enzyme homologs (e.g., Glu156/Gly166 minus Gln156(Q156)/Gly166) reflects the change in catalytic efficiency that may be attributed solely to electrostatic effects.

These results show that the average change in substrate preference is considerably greater when electrostatic substitutions are produced at position 166 (50-fold in kcat/Km) versus position 156 (12-fold in kcat/Km). From these ΔΔlog kcat/Km values, an average change in transition-state stabilization energy can be calculated of -1.5 and -2.4 kcal/mol for substitutions at positions 156 and 166, respectively. This should represent the stabilization energy contributed from a favorable electrostatic interaction for the binding of free enzyme and substrate to form the transition-state complex.

EXAMPLE 10

Substitutions at Position 217

Tyr217 has been substituted by all other 19 amino acids. Cassette mutagenesis as described in EPO publication No. 0130756 was used according to the protocol of Figure 22. The EcoRV restriction site was used for restriction-purification of pΔ217.

Since this position is involved in substrate binding, mutations here effect kinetic parameters of the enzyme. An example is the substitution of Leu for Tyr at position 217. For the substrate sAAPFpNa, this mutant has a kcat of 277 5' and a Km of 4.7×10^{-4} with a kcat/Km ratio of 6×10^{5}. This represents a 5.5-fold increase in kcat with a 3-fold increase in Km over the wild type enzyme.

In addition, replacement of Tyr217 by Lys, Arg, Phe or Leu results in mutant enzymes which are more stable at pHs of about 9-11 than the WT enzyme. Conversely, replacement of Tyr217 by Asp, Glu, Gly or Pro results in enzymes which are less stable at pHs of about 9-11 than the WT enzyme.

EXAMPLE 11

Multiple Mutants Having Altered Thermal Stability

B. amyloliquefacien subtilisin does not contain any cysteine residues. Thus, any attempt to produce thermal stability by Cys cross-linkage required the substitution of more than one amino acid in subtilisin with Cys. The following subtilisin residues were multiply substituted with cysteine:

Thr22/Ser87

Ser24/Ser87

Mutagenesis of Ser24 to Cys was carried out with a 5' phosphorylated oligonucleotide primer having the sequence

5'-pC-TAC-ACT-GGA-TGC-AAT-GTT-AAA-G-3'.

(Asterisks show the location of mismatches and the underlined sequence shows the position of the altered Sau3A site.) The B. amyloliquefaciens subtilisin gene on a 1.5 kb EcoRI-BAMHI fragment from pS4.5 was cloned into M13mp11 and single stranded DNA was isolated. This template (M13mp11SUBT) was double primed with the 5' phosphorylated M13 universal sequencing primer and the mutagenesis primer. Adelman, et al. (1983) DNA 2, 183-193. The heteroduplex was transfected into competent JM101 cells and plaques were probed for the mutant sequence (Zoller, M.J., et al. (1982) Nucleic Acid Res. 10, 6487-6500; Wallace, et al. (1981) Nucleic Acid Res. 9, 3647-3656) using a tetramethylammonium chloride hybridization protocol (Wood, et al. (1985) Proc. Natl. Acad. Sci. USA 82, 1585-1588). The Ser87 to Cys mutation was prepared in a similar fashion using a 5' phosphorylated primer having the sequence

5'-pGGC-GTT-GCG-CCA-TGC-GCA-TCA-CT-3'.

(The asterisk indicates the position of the mismatch and the underlined sequence shows the position of a new MstI site.) The C24 and C87 mutations were obtained at a frequency of one and two percent, respectively. Mutant sequences were confirmed by dideoxy sequencing in M13.

Mutagenesis of Tyr21/Thr22 to A21/C22 was carried out with a 5' phosphorylated oligonucleotide primer having the sequence

5'-pAC-TCT-CAA-GGC-GCT-TGT-GGC-TCA-AAT-GTT-3'.

(The asterisks show mismatches to the wild type sequence and the underlined sequence shows the position of an altered Sau3A site.) Manipulations for heteroduplex synthesis were identical to those described for C24. Because direct cloning of the heteroduplex DNA fragment can yield increased frequencies of mutagenesis, the EcoRI-BamHI subtilisin fragment was purified and ligated into pBS42. E. coli MM 294 cells were transformed with the ligation mixture and plasmid DNA was purified from isolated transformants. Plasmid DNA was screened for the loss of the Sau3A site at codon 23 that was eliminated by the mutagenesis primer. Two out of 16 plasmid preparations had lost the wild type Sau3A site. The mutant sequence was confirmed by dideoxy sequencing in M13.

Double mutants, C22/C87 and C24/C87, were constructed by ligating fragments sharing a common ClaI site that separated the single parent cystine codons. Specifically, the 500 bp EcoRI-ClaI fragment containing the 5' portion of the subtilisin gene (including codons 22 and 24) was ligated with the 4.7 kb ClaI-EcoRI fragment that contained the 3' portion of the subtilisin gene (including codon 87) plus pBS42 vector sequence. E. coli MM 294 was transformed with ligation mixtures and plasmid DNA was purified from individual transformants. Double-cysteine plasmid constructions were identified by restriction site markers originating from the parent cysteine mutants (i.e., C22 and C24, Sau3A minus; Cys87, MstI plus). Plasmids from E. coli were transformed into B. subtilis BG2036. The thermal stability of these mutants as compared to wild type subtilisin are presented in Figure 30 and Tables XVII and XVIII.

TABLE XVII

Effect of DTT on the Half-Time of Autolytic Inactivation of Wild-Type and Disulfide Mutants of Subtilisin*			
Enzyme	$t_{\frac{1}{2}}$		-DTT/+DTT
	-DDT	+DTT	
	min		
Wild-type	95	85	1.1
C22/C87	44	25	1.8
C24/C87	92	62	1.5

(*) Purified enzymes were either treated or not treated with 25mM DTT and dialyzed with or without 10mM DTT in 2mM $CaCl_2$, 50mM Tris (pH 7.5) for 14 hr. at 4°C. Enzyme concentrations were adjusted to 80μl aliquots were quenched on ice and assayed for residual activity. Half-times for autolytic inactivation were determined from semi-log plots of $\log_{10}$ (residual activity) versus time. These plots were linear for over 90% of the inactivation.

TABLE XVIII

Effect of Mutations in Subtilisin on the Half-Time of Autolytic Inactivation at 58°C*	
Enzyme	$t_{\frac{1}{2}}$
	min
Wild-type	120
C22	22
C24	120
C87	104
C22/C87	43
C24/C87	115

(*) Half-times for autolytic inactivation were determined for wild-type and mutant subtilisins as described in the legend to Table III. Unpurified and non-reduced enzymes were used directly from B. subtilis culture supernatants.

The disulfides introduced into subtilisin did not improve the autolytic stability of the mutant enzymes when compared to the wild-type enzyme. However, the disulfide bonds did provide a margin of autolytic stability when compared to their corresponding reduced double-cysteine enzyme. Inspection of a highly refined x-ray structure of wild-type B. amyloliquefaciens subtilisin reveals a hydrogen bond between Thr22 and Ser87. Because cysteine is a poor hydrogen donor or acceptor (Paul, I.C. (1974) in Chemistry of the -SH Group (Patai, S., ed.) pp. 111-149, Wiley Interscience, New York) weakening of 22/87 hydrogen bond may explain why the C22 and C87 single-cysteine mutant proteins are less autolytically stable than either C24 or wild-type (Table XVIII). The fact that C22 is less autolytically stable than C87 may be the result of the Tyr21A mutation (Table XVIII). Indeed, construction and analysis of Tyr21/C22 shows the mutant protein has an autolytic stability closer to that of C87. In summary, the C22 and C87 of single-cysteine mutations destabilize the protein toward autolysis, and disulfide bond formation increases the stability to a level less than or equal to that of wild-type enzyme.

EXAMPLE 12

Multiple Mutants Containing Substitutions at Position 222 and Position 166 or 169

Double mutants 166/222 and 169/222 were prepared by ligating together (1) the 2.3kb AcaII fragment from pS4.5 which contains the 5' portion of the subtilisin gene and vector sequences, (2) the 200bp AvaII fragment which contains the relevant 166 or 169 mutations from the respective 166 or 169 plasmids, and (3) the 2.2kb AvaII fragment which contains the relevant 222 mutation 3' and of the subtilisin genes and vector

sequence from the respective p222 plasmid.

Although mutations at position 222 improve oxidation stability they also tend to increase the Km. An example is shown in Table XIX. In this case the A222 mutation was combined with the K166 mutation to give an enzyme with kcat and Km intermediate between the two parent enzymes.

TABLE XIX

	kcat	Km
WT	50	1.4×10^{-4}
A222	42	9.9×10^{-4}
K166	21	3.7×10^{-5}
K166/A222	29	2.0×10^{-4}
substrate sAAPFpNa		

EXAMPLE 13

Multiple Mutants Containing Substitutions at Positions 50, 156, 166, 217 and Combinations Thereof

The double mutant S156/A169 was prepared by ligation of two fragments, each containing one of the relevant mutations. The plasmid pS156 was cut with XmaI and treated with S1 nuclease to create a blunt end at codon 167. After removal of the nuclease by phenol/chloroform extraction and ethanol precipitation, the DNA was digested with BamHI and the approximately 4kb fragment containing the vector plus the 5' portion of the subtilisin gene through codon 167 was purified.

The pA169 plasmid was digested with KpnI and treated with DNA polymerase Klenow fragment plus 50 µM dNTPs to create a blunt end codon at codon 168. The Klenow was removed by phenol/chloroform extraction and ethanol precipitation. The DNA was digested with BamHI and the 590bp fragment including codon 168 through the carboxy terminus of the subtilisin gene was isolated. The two fragments were then ligated to give S156/A169.

Triple and quadruple mutants were prepared by ligating together (1) the 220bp PvuII/HaeII fragment containing the relevant 156, 166 and/or 169 mutations from the respective p156, p166 and/or p169 double of single mutant plasmid, (2) the 550bp HaeII/BamHI fragment containing the relevant 217 mutant from the respective p217 plasmid, and (3) the 3.9kb PvuII/BamHI fragment containing the F50 mutation and vector sequences.

The multiple mutant F50/S156/A169/L217, as well as B. amyloliquefaciens subtilisin, B. lichenformis subtilisin and the single mutant L217 were analyzed with the above synthetic polypeptides where the P-1 amino acid in the substrate was Lys, His, Ala, Gln, Tyr, Phe, Met and Leu. These results are shown in Figures 26 and 27.

These results show that the F50/S156/A169/L217 mutant has substrate specificity similar to that of the B. licheniformis enzyme and differs dramatically from the wild type enzyme. Although only data for the L217 mutant are shown, none of the single mutants (e.g., F50, S156 or A169) showed this effect. Although B. licheniformis differs in 88 residue positions from B. amyloliquefaciens, the combination of only these four mutations accounts for most of the differences in substrate specificity between the two enzymes.

EXAMPLE 14

Subtilisin Mutants Having Altered Alkaline Stability

A random mutagenesis technique was used to generate single and multiple mutations within the B. amyloliquefaciens subtilisin gene. Such mutants were screened for altered alkaline stability. Clones having increased (positive) alkaline stability and decreased (negative) alkaline stability were isolated and sequenced to identify the mutations within the subtilisin gene. Among the positive clones, the mutants V107 and R213 were identified. These single mutants were subsequently combined to produce the mutant V107/R213.

One of the negative clones (V50) from the random mutagenesis experiments resulted in a marked decrease in alkaline stability. Another mutant (P50) was analyzed for alkaline stability to determine the effect

of a different substitution at position 50. The F50 mutant was found to have a greater alkaline stability than wild type subtilisin and when combined with the double mutant V107/R213 resulted in a mutant having an alkaline stability which reflected the aggregate of the alkaline stabilities for each of the individual mutants.

The single mutant R204 and double mutant C204/R213 were identified by alkaline screening after random cassette mutagenesis over the region from position 197 to 228. The C204/R213 mutant was thereafter modified to produce mutants containing the individual mutations C204 and R213 to determine the contribution of each of the individual mutations. Cassette mutagenesis using pooled oligonucleotides to substitute all amino acids at position 204, was utilized to determine which substitution at position 204 would maximize the increase in alkaline stability. The mutation from Lys213 to Arg was maintained constant for each of these substitutions at position 204.

A. Construction of pB0180, an E. coli-B. subtilis Shuttle Plasmid

The 2.9 kb EcoRI-BamHI fragment from pBR327 (Covarrubias, L., et al. (1981) Gene 13, 25-35) was ligated to the 3.7kb EcoRI-BamHI fragment of pBD64 (Gryczan, T., et al. (1980) J. Bacteriol., 141, 246-253) to give the recombinant plasmid pB0153. The unique EcoRI recognition sequence in pBD64 was eliminated by digestion with EcoRI followed by treatment with Klenow and deoxynucleotide triphosphates (Maniatis, T., et al. (eds.) (1982) in Molecular Cloning, A Laboratory Manual, Cold spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Blunt end ligation and transformation yielded pB0154. The unique AvaI recognition sequence in pBO154 was eliminated in a similar manner to yield pBO171. pB0171 was digested with BamHI and PvuII and treated with Klenow and deoxynucleotide triphosphates to create blunt ends. The 6.4 kb fragment was purified, ligated and transformed into LE392 cells (Enquest, L.W., et al. (1977) J. Mol. Biol. 111, 97-120), to yield pB0172 which retains the unique BamHI site. To facilitate subcloning of subtilisin mutants, a unique and silent KpnI site starting at codon 166 was introduced into the subtilisin gene from pS4.5 (Wells, J.A., et al. (1983) Nucleic Acids Res., 11, 7911-7925) by site-directed mutagenesis. The KpnI+ plasmid was digested with EcoRI and treated with Klenow and deoxynucleotide triphosphates to create a blunt end. The Klenow was inactivated by heating for 20 min at 68°C, and the DNA was digested with BamHI. The 1.5 kb blunt EcoRI-BamHI fragment containing the entire subtilisin was ligated with the 5.8 kb NruI-BamHI from pB0172 to yield pBO180. The ligation of the blunt NruI end to the blunt EcoRI end recreated an EcoRI site. Proceeding clockwise around pB0180 from the EcoRI site at the 5' end of the subtilisin gene is the unique BamHI site at the 3' end of the subtilisin gene, the chloramphenicol and neomycin resistance genes and UB110 gram positive replication origin derived from pBD64, the ampicillin resistance gene and gram negative replication origin derived from pBR327.

B. Construction of Random Mutagenesis Library

The 1.5 kb EcoRI-BamHI fragment containing the B. amyloliquefaciens subtilisin gene (Wells et al., 1983) from pB0180 was cloned into M13mp11 to give M13mp11 SUBT essentially as previously described (Wells, J.A., et al. (1986) J. Biol. Chem., 261,6564-6570). Deoxyuridine containing template DNA was prepared according to Kunkel (Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA, 82 488-492). Uridine containing template DNA (Kunkel, 1985) was purified by CsCl density gradients (Maniatis, T. et al. (eds.) (1982) in Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). A primer (AvaI⁻) having the sequence

$$5'\text{GAAAAAAGA}\underline{\text{CCC}\overset{*}{\text{T}}\text{AG}}\text{CGTCGCTTA}$$

ending at codon -11, was used to alter the unique AvaI recognition sequence within the subtilisin gene. (The asterisk denotes the mismatches from the wild-type sequence and underlined is the altered AvaI site.)

The 5' phosphorylated AvaI primer (~320 pmol) and ~40 pmol (~120µg) of uridine containing M13mp11 SUBT template in 1.88 ml of 53 mM NaCl, 7.4 mM MgCl2 and 7.4 mM Tris.HCl (pH 7.5) were annealed by heating to 90°C for 2 min. and cooling 15 min at 24°C (Fig. 31). Primer extension at 24°C was initiated by addition of 100µL containing 1 mM in all four deoxynucleotide triphosphates, and 20µl Klenow fragment (5 units/l). The extension reaction was stopped every 15 seconds over ten min by addition of 10µl 0.25 M EDTA (pH 8) to 50µl aliquots of the reaction mixture. Samples were pooled, phenol chlorophorm extracted and DNA was precipitated twice by addition of 2.5 vol 100% ethanol, and washed twice with 70% ethanol.

The pellet was dried, and redissolved in 0.4 ml 1 mM EDTA, 10 mM Tris (pH 8).

Misincorporation of α-thiodeoxynucleotides onto the 3' ends of the pool of randomly terminated template was carried out by incubating four 0.2 ml solutions each containing one-fourth of the randomly terminated template mixture (~20µg), 0.25 mM of a given α-thiodeoxynucleotide triphosphate, 100 units AMV polymerase, 50 mM KCL, 10 mM $MgCl_2$, 0.4 mM dithiothreitol, and 50 mM Tris (pH 8.3) (Champoux, J.J. (1984) Genetics, 2, 454-464). After incubation at 37°C for 90 minutes, misincorporation reactions were sealed by incubation for five minutes at 37°C with 50 mM all four deoxynucleotide triphosphates (pH 8), and 50 units AMV polymerase. Reactions were stopped by addition of 25 mM EDTA (final), and heated at 68°C for ten min to inactivate AMV polymerase. After ethanol precipitation and resuspension, synthesis of closed circular heteroduplexes was carried out for two days at 14°C under the same conditions used for the timed extension reactions above, except the reactions also contained 1000 units T4 DNA ligase, 0.5 mM ATP and 1 mM β-mercaptoethanol. Simultaneous restriction of each heteroduplex pool with KpnI, BamHI, and EcoRI confirmed that the extension reactions were nearly quantitative. Heteroduplex DNA in each reaction mixture was methylated by incubation with 80µM S-adenosylmethionine and 150 units dam methylase for 1 hour at 37°C. Methylation reactions were stopped by heating at 68°C for 15 min.

One-half of each of the four methylated heteroduplex reactions were transformed into 2.5 ml competent E. coli JM101 (Messing, J. (1979) Recombinant DNA Tech. Bull., 2, 43-48). The number of independent transformants from each of the four transformations ranged from 0.4-2.0 x 10^5. After growing out phage pools, RF DNA from each of the four transformations was isolated and purified by centrifugation through CsCl density gradients. Approximately 2µg of RF DNA from each of the four pools was digested with EcoRI, BamHI and AvaI. The 1.5 kb EcoRI-BamHI fragment (i.e., AvaI resistant) was purified on low gel temperature agarose and ligated into the 5.5 kb EcoRI-BamHI vector fragment of pB0180. The total number of independent transformants from each α-thiodeoxynucleotide misincorporation plasmid library ranged from 1.2-2.4 x 10^4. The pool of plasmids from each of the four transformations was grown out in 200 ml LB media containing 12.5µg/ml cmp and plasmid DNA was purified by centrifugation through CsCl density gradients.

C. Expression and Screening of Subtilisin Point Mutants

Plasmid DNA from each of the four misincorporation pools was transformed (Anagnostopoulos, C., et al. (1967), J. Bacteriol., 81, 741-746) into BG2036. For each transformation, 5µg of DNA produced approximately 2.5 x 10^5 independent BG2036 transformants, and liquid culture aliquots from the four libraries were stored in 10% glycerol at 70°C. Thawed aliquots of frozen cultures were plated on LB/5µg/ml cmp/1.6% skim milk plates (Wells, J.A., et al. (1983) Nucleic Acids Res., 11, 7911-7925), and fresh colonies were arrayed onto 96-well microtiter plates containing 150 l per well LB media plus 12.5µg/ml cmp. After 1 h at room temperature, a replica was stamped (using a matched 96 prong stamp) onto a 132 mm BA 85 nitrocellulose filter (Schleicher and Scheull) which was layered on a 140 mm diameter LB/cmp/skim milk plate. Cells were grown about 16 h at 30°C until halos of proteolysis were roughly 5-7 mm in diameter and filters were transferred directly to a freshly prepared agar plate at 37°C containing only 1.6% skim milk and 50 mM sodium phosphate pH 11.5. Filters were incubated on plates for 3-6 h at 37°C to produce halos of about 5 mm for wild-type subtilisin and were discarded. The plates were stained for 10 min at 24°C with Coomassie blue solution (0.25% Coomassie blue (R-250) 25% ethanol) and destained with 25% ethanol, 10% acetic acid for 20 min. Zones of proteolysis appeared as blue halos on a white background on the underside of the plate and were compared to the original growth plate that was similarly stained and destained as a control. Clones were considered positive that produced proportionately larger zones of proteolysis on the high pH plates relative to the original growth plate. Negative clones gave smaller halos under alkaline conditions. Positive and negative clones were restreaked to colony purify and screened again in triplicate to confirm alkaline pH results.

D. Identification and Analysis of Mutant Subtilisins

Plasmid DNA from 5 ml overnight cultures of more alkaline active B.subtilis clones was prepared according to Birnboim and Doly (Birnboim, H.C., et al. (1979) Nucleic Acid Res. 7, 1513) except that incubation with 2 mg/ml lysozyme proceeded for 5 min at 37°C to ensure cell lysis and an additional phenol/$CHCl_3$ extraction was employed to remove contaminants. The 1.5 kb EcoRI-BamHI fragment containing the subtilisin gene was ligated into M13mp11 and template DNA was prepared for DNA sequencing (Messing, J., et al. (1982) Gene, 19 269-276). Three DNA sequencing primers ending at codon 26, +95, and +155 were synthesized to match the subtilisin coding sequence. For preliminary sequence

identification a single track of DNA sequence, corresponding to the dNTPaS misincorporation library from which the mutant came, was applied over the entire mature protein coding sequence (i.e., a single dideoxyguanosine sequence track was applied to identify a mutant from the dGTPas library). A complete four track of DNA sequence was performed 200 bp over the site of mutagenesis to confirm and identify the mutant sequence (Sanger, F., et al., (1980) J. Mol. Biol., 143, 161-178). Confirmed positive and negative bacilli clones were cultured in LB media containing 12.5μg/mL cmp and purified from culture supernatants as previously described (Estell, D.A., et al. (1985) J. Biol. Chem., 260, 6518-6521). Enzymes were greater than 98% pure as analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, U.K. (1970), Nature, 227, 680-685), and protein concentrations were calculated from the absorbance at 280 nm,

$$\varepsilon_{280}^{0.1\%} = 1.17$$

(Maturbara, H., et al. (1965), J. Biol. Chem, 240, 1125-1130).

Enzyme activity was measured with 200μg/mL succinyl-L-AlaL-AlaL-ProL-Phep-nitroanilide (Sigma) in 0.1M Tris pH 8.6 or 0.1 M CAPS pH 10.8 at 25 °C. Specific activity (μ moles product/min-mg) was calculated from the change in absorbance at 410 nm from production of p-nitroaniline with time per mg of enzyme (E410 = 8,480 M-lcm-l; Del Mar, E.G., et al. (1979), Anal. Biochem., 99, 316-320). Alkaline autolytic stability studies were performed on purified enzymes (200μg/mL) in 0.1 M potassium phosphate (pH 12.0) at 37 °C. At various times aliquots were assayed for residual enzyme activity (Wells, J.A., et al. (1986) J. Biol. Chem., 261, 6564-6570).

E. Results

1. Optimization and analysis of mutagenesis frequency

A set of primer-template molecules that were randomly 3'-terminated over the subtilisin gene (Fig. 31) was produced by variable extension from a fixed 5'-primer (The primer mutated a unique AvaI site at codon 11 in the subtilisin gene). This was achieved by stopping polymerase reactions with EDTA after various times of extension. The extent and distribution of duplex formation over the 1 kb subtilisin gene fragment was assessed by multiple restriction digestion (not shown). For example, production of new HinfI fragments identified when polymerase extension had proceeded past Ile110, Leu233, and Asp259 in the subtilisin gene.

Misincorporation of each dNTPαs at randomly terminated 3' ends by AMV reverse transcriptase (Zakour, R.A., et al. (1982), Nature, 295, 708-710; Zakour, R.A., et al. (1984), Nucleic Acids Res., 12, 6615-6628) used conditions previously described (Champoux, J.J., (1984), Genetics, 2, 454-464). The efficiency of each misincorporation reaction was estimated to be greater than 80% by the addition of each dNTPαs to the AvaI restriction primer, and analysis by polyacrylamide gel electrophoresis. Misincorporations were sealed by polymerization with all four dNTP's and closed circular DNA was produced by reaction with DNA ligase.

Several manipulations were employed to maximize the yield of the mutant sequences in the heteroduplex. These included the use of a deoxyuridine containing template (Kunkel, T.A. (1985), Proc. Natl. Acad. Sci. USA, 82 488-492; Pukkila, P.J. et al. (1983), Genetics, 104, 571-582), in vitro methylation of the mutagenic strand (Kramer, W. et al. (1982) Nucleic Acids Res., 10 6475-6485), and the use of AvaI restriction-selection against the wild-type template strand which contained a unique AvaI site. The separate contribution of each of these enrichment procedures to the final mutagenesis frequency was not determined, except that prior to AvaI restriction-selection roughly one-third of the segregated clones in each of the four pools still retained a wild-type AvaI site within the subtilisin gene. After AvaI restriction-selection greater than 98% of the plasmids lacked the wild-type AvaI site.

The 1.5 kb EcoRI-BamHI subtilisin gene fragment that was resistant to AvaI restriction digestion, from each of the four CsCl purified M13 RF pools was isolated on low melting agarose. The fragment was ligated in situ from the agarose with a similarly cut E. coli-B. subtilis shuttle vector, pB0180, and transformed directly into E coli LE392. Such direct ligation and transformation of DNA isolated from agarose avoided loses and allowed large numbers of recombinants to be obtained (>100,000 per μg equivalent of input M13 pool).

The frequency of mutagenesis for each of the four dNTPαs misincorporation reactions was estimated from the frequency that unique restriction sites were eliminated (Table XX). The unique restriction sites

chosen for this analysis, ClaI, PvuII, and KpnI, were distributed over the subtilisin gene starting at codons 35, 104, and 166, respectively. As a control, the mutagenesis frequency was determined at the PstI site located in the β lactamase gene which was outside the window of mutagenesis. Because the absolute mutagenesis frequency was close to the percentage of undigested plasmid DNA, two rounds of restriction-selection were necessary to reduce the background of surviving uncut wild-type plasmid DNA below the mutant plasmid (Table XX). The background of surviving plasmid from wild-type DNA probably represents the sum total of spontaneous mutations, uncut wild-type plasmid, plus the efficiency with which linear DNA can transform E. coli. Subtracting the frequency for unmutagenized DNA (background) from the frequency for mutant DNA, and normalizing for the window of mutagenesis sampled by a given restriction analysis (4-6 bp) provides an estimate of the mutagenesis efficiency over the entire coding sequence (~1000 bp).

TABLE XX

α-thiol dNTP misincorporated(b)	Restriction Site Selection	% resistant clones[c] 1st round	2nd round	Total	% resistant clones over Background[d]	% mutants per 1000bp[e]
None	PstI	0.32	0.7	0.002	0	-
G	PstI	0.33	1.0	0.003	0.001	0.2
T	PstI	0.32	<0.5	<0.002	0	0
C	PstI	0.43	3.0	0.013	0.011	3
None	ClaI	0.28	5	0.014	0	-
G	ClaI	2.26	85	1.92	1.91	380
T	ClaI	0.48	31	0.15	0.14	35
C	ClaI	0.55	15	0.08	0.066	17
None	PvuII	0.08	29	0.023	0	-
G	PvuII	0.41	90	0.37	0.35	88
T	PvuII	0.10	67	0.067	0.044	9
C	PvuII	0.76	53	0.40	0.38	95
None	KpnI	0.41	3	0.012	0	-
G	KpnI	0.98	35	0.34	0.33	83
T	KpnI	0.36	15	0.054	0.042	8
C	KpnI	1.47	26	0.38	0.37	93

(a) Mutagenesis frequency is estimated from the frequency for obtaining mutations that alter unique restriction sites within the mutagenized subtilisin gene (i.e., ClaI, PvuII, or KpnI) compared to mutation frequencies of the PstI site, that is outside the window of mutagenesis.

(b) Plasmid DNA was from wild-type (none) or mutagenized by dNTPαs misincorporation as described.

(c) Percentage of resistant clones was calculated from the fraction of clones obtained after three fold or greater over-digestion of the plasmid with the indicated restriction enzyme compared to a

non-digested control. Restriction-resistant plasmid DNA from the first round was subjected to a second round of restriction-selection. The total represents the product of the fractions of resistant clones obtained from both rounds of selection and gives percentage of restriction-site mutant clones in the original starting pool. Frequencies were derived from counting at least 20 colonies and usually greater than 100.

(d) Percent resistant clones was calculated by subtracting the percentage of restriction-resistant clones obtained for wild-type DNA (i.e., none) from that obtained for mutant DNA.

(e) This extrapolates from the frequency of mutation over each restriction site to the entire subtilisin gene (~1 kb). This has been normalized to the number of possible bases (4-6 bp) within each restriction site that can be mutagenized by a given misincorporation event.

From this analysis, the average percentage of subtilisin genes containing mutations that result from dGTPαs, dCTPαs, or dTTPαs misincorporation was estimated to be 90, 70, and 20 percent, respectively. These high mutagenesis frequencies were generally quite variable depending upon the dNTPαs and misincorporation efficiencies at this site. Misincorporation efficiency has been reported to be both dependent on the kind of mismatch, and the context of primer (Champoux, J.J., (1984); Skinner, J.A., et al. (1986) Nucleic Acids Res., 14, 6945-6964). Biased misincorporation efficiency of dGTPαs and dCTPαs over dTTPαs has been previously observed (Shortle, D., et al. (1985), Genetics, 110, 539-555). Unlike the dGTPαs, dCTPαs, and dTTPαs libraries the efficiency of mutagenesis for the dATPαs misincorporation library could not be accurately assessed because 90% of the restriction-resistant plasmids analyzed simply lacked the subtilisin gene insert. This problem probably arose from self-ligation of the vector when the dATPαs mutagenized subtilisin gene was subcloned from M13 into pB0180. Correcting for the vector background, we estimate the mutagenesis frequency around 20 percent in the dATPαs misincorporation library. In a separate experiment (not shown), the mutagenesis efficiencies for dGTPαs and dTTPαs misincorporation were estimated to be around 50 and 30 percent, respectively, based on the frequency of reversion of an inactivating mutation at codon 169.

The location and identity of each mutation was determined by a single track of DNA sequencing corresponding to the misincorporated αthiodeoxynucleotide over the entire gene followed by a complete four track of DNA sequencing focused over the site of mutation. Of 14 mutants identified, the distribution was similar to that reported by Shortle and Lin (1985) except we did not observe nucleotide insertion or deletion mutations. The proportion of AG mutations was highest in the G misincorporation library, and some unexpected point mutations appeared in the dTTPαs and dCTPαs libraries.

2. Screening and Identification of Alkaline Stability Mutants of Subtilisin

It is possible to screen colonies producing subtilisin by halos of casein digestion (Wells, J.A. et al. (1983) Nucleic Acids Res., 11, 7911-7925). However, two problems were posed by screening colonies under high alkaline conditions (>pH 11). First, B. subtilis will not grow at high pH, and we have been unable to transform an alkylophilic strain of bacillus. This problem was overcome by adopting a replica plating strategy in which colonies were grown on filters at neutral pH to produce subtilisin and filters subsequently transferred to casein plates at pH 11.5 to assay subtilisin activity. However, at pH 11.5 the casein micells no longer formed a turbid background and thus prevented a clear observation of proteolysis halos. The problem was overcome by briefly staining the plate with Coomassie blue to amplify proteolysis zones and acidifying the plates to develop casein micell turbidity. By comparison of the halo size produced on the reference growth plate (pH 7) to the high pH plate (pH 11.5), it was possible to identify mutant subtilisins that had increased (positives) or decreased (negatives) stability under alkaline conditions.

Roughly 1000 colonies were screened from each of the four misincorporation libraries. The percentage of colonies showing a differential loss of activity at pH 11.5 versus pH 7 represented 1.4, 1.8, 1.4, and 0.6% of the total colonies screened from the thiol dGTPαs, dATPαs, dTTPαs, and dCTPαs libraries, respectively. Several of these negative clones were sequenced and all were found to contain a single base change as expected from the misincorporation library from which they came. Negative mutants included A36, E170 and V50. Two positive mutants were identified as V107 and R213. The ratio of negatives to positives was roughly 50:1.

3. Stability and Activity of Subtilisin Mutants at Alkaline pH

Subtilisin mutants were purified and their autolytic stabilities were measured by the time course of inactivation at pH 12.0 (Figs. 32 and 33). Positive mutants identified from the screen (i.e., V107 and R213) were more resistant to alkaline induced autolytic inactivation compared to wild-type; negative mutants (i.e., E170 and V50) were less resistant. We had advantageously produced another mutant at position 50 (F50) by site-directed mutagenesis. This mutant was more stable than wild-type enzyme to alkaline autolytic inactivation (Fig. 33) At the termination of the autolysis study, SDS-PAGE analysis confirmed that each subtilisin variant had autolyzed to an extent consistent with the remaining enzyme activity.

The stabilizing effects of V107, R213, and F50 are cumulative. See Table XXI. The double mutant, V107/R213 (made by subcloning the 920 bp EcoRI-KpnI fragment of pB0180V107 into the 6.6 kb EcoRI-KpnI fragment of pB0180R213), is more stable than either single mutant. The triple mutant, F50/V107/R213 (made by subcloning the 735 bp EcoRI-PvuII fragment of pF50 (Example 2) into the 6.8 kb EcoRI-PvuII fragment of pB0180/V107, is more stable than the double mutant V107/R213 or F50. The inactivation curves show a biphasic character that becomes more pronounced the more stable the mutant analyzed. This may result from some destablizing chemical modification(s) (eg., deamidation) during the autolysis study and/or reduced stabilization caused by complete digestion of larger autolysis peptides. These alkaline autolysis studies have been repeated on separately purified enzyme batches with essentially the same results. Rates of autolysis should depend both on the conformational stability as well as the specific activity of the subtilisin variant (Wells, J.A., et al. (1986), J. Biol. Chem., 261, 6564-6570). It was therefore possible that the decreases in autolytic inactivation rates may result from decreases in specific activity of the more stable mutant under alkaline conditions. In general the opposite appears to be the case. The more stable mutants, if anything, have a relatively higher specific activity than wild-type under alkaline conditions and the less stable mutants have a relatively lower specific activity. These subtle effects on specific activity for V107/R213 and F50/V107/R213 are cumulative at both pH 8.6 and 10.8. The changes in specific activity may reflect slight differences in substrate specificity, however, it is noteworthy that only positions 170 and 107 are within 6A of a bound model substrate (Robertus, J.D., et al. (1972), Biochemistry 11, 2438-2449).

TABLE XXI

Relationship between relative specific acitivity at pH 8.6 or 10.8 and alkaline autolytic stability			
Enzyme	Relative specific activity		Alkaline autolysis half-time (min)b
	pH 8.6	pH 10.8	
Wild-type	100±1	100±3	86
Q170	46±1	28±2	13
V107	126±3	99±5	102
R213	97±1	102±1	115
V107/R213	116±2	106±3	130
V50	66±4	61±1	58
F50	123±3	157±7	131
F50/V107/R213	126±2	152±3	168

(a) Relative specific activity was the average from triplicate activity determinations divided by the wild-type value at the same pH. The average specific activity of wild-type enzyme at pH 8.6 and 10.8 was 70µmoles/min-mg and 37µmoles/min-mg, respectively.
(b) Time to reach 50% activity was taken from Figs. 32 and 33.

F. Random Cassette Mutagenesis of Residues 197 through 228

Plasmid pΔ222 (Wells, et al. (1985) Gene 34, 315-323) was digested with PstI and BamHI and the 0.4 kb PstI/BamHI fragment (fragment 1, see Fig. 34) purified from a polyacrylamide gel by electroelution.

The 1.5 kb EcoRI/BamHI fragment from pS4.5 was cloned into M13mp9. Site directed mutagenesis was performed to create the A197 mutant and simultaneously insert a silent SstI site over codons 195-196. The mutant EcoRI/BamHI fragment was cloned back into pBS42. The pA197 plasmid was digested with BamHI and SstI and the 5.3 kb BamHI/SstI fragment (fragment 2) was purified from low melting agarose.

Complimentary oligonucleotides were synthesized to span the region from SstI (codons 195-196) to PstI (codons 228-230). These oligodeoxynucleotides were designed to (1) restore codon 197 to the wild type, (2) re-create a silent KpnI site present in pΔ222 at codons 219-220, (3) create a silent SmaI site over codons 210-211, and (4) eliminate the PstI site over codons 228-230 (see Fig. 35). Oligodeoxynucleotides were synthesized with 2% contaminating nucleotides at each cycle of synthesis, e.g., dATP reagent was spiked with 2% dCTP, 2% dGTP, and 2% dTTP. For 97-mers, this 2% poisoning should give the following percentages of non-mutant, single mutants and double or higher mutants per strand with two or more misincorporations per complimentary strand: 14% non-mutant, 28% single mutant, and 57% with ≥2 mutations, according to the general formula

$$f = \frac{\mu^n}{n!} e^{-\mu} .$$

where μ is the average number of mutations and n is a number class of mutations and f is the fraction of the total having that number of mutations. Complimentary oligodeoxynucleotide pools were phosphorylated and annealed (fragment 3) and then ligated at 2-fold molar excess over fragments 1 and 2 in a three-way ligation.

E. coli MM294 was transformed with the ligation reaction, the transformation pool-grown up over night and the pooled plasmid DNA was isolated. This pool represented 3.4 x 10^4 independent transformants. This plasmid pool was digested with PstI and then used to retransform E. coli. A second plasmid pool was prepared and used to transform B. subtilis (BG2036). Approximately 40% of the BG2036 transformants actively expressed subtilisin as judged by halo-clearing on casein plates. Several of the non-expressing transformants were sequenced and found to have insertions or deletions in the synthetic cassettes. Expressing BG2036 mutants were arrayed in microtiter dishes with 150µl of LB/12.5µg/mL chloramphenicol (cmp) per well, incubated at 37°C for 3-4 hours and then stamped in duplicate onto nitrocellulose filters laid on LB 1.5% skim milk/5µg/mL cmp plates and incubated overnight at 33°C (until halos were approximately 4-8 mm in diameter). Filters were then lifted to stacks of filter paper saturated with 1 x Tide commercial grade detergent, 50 mM Na_2CO_3, pH 11.5 and incubated at 65°C for 90 min. Overnight growth plates were Commassie stained and destained to establish basal levels of expression. After this treatment, filters were returned to pH7/skim milk/20µg/mL tetracycline plates and incubated at 37°C for 4 hours to overnight.

Mutants identified by the high pH stability screen to be more alkaline stable were purified and analyzed for autolytic stability at high pH or high temperature. The double mutant C204/R213 was more stable than wild type at either high pH or high temperature (Table XXII).

This mutant was dissected into single mutant parents (C204 and R213) by cutting at the unique SmaI restriction site (Fig. 35) and either ligating wild type sequence 3' to the SmaI site to create the single C204 mutant or ligating wild type sequence 5' to the SmaI site to create the single R213 mutant. Of the two single parents, C204 was nearly as alkaline stable as the parent double mutant (C04/R213) and slightly more thermally stable. See Table XXII. The R213 mutant was only slightly more stable than wild type under both conditions (not shown).

Another mutant identified from the screen of the 197 to 228 random cassette mutagenesis was R204. This mutant was more stable than wild type at both high pH and high temperature but less stable than C204.

TABLE XXII

Stability of subtilisin variants

Purified enzymes (200µg/mL) were incubated in 0.1M phosphate, pH 12 at 30°C for alkaline autolysis, or in 2mM $CaCl_2$, 50mM MOPS, pH 7.0 at 62°C for thermal autolysis. At various times samples were assayed for residual enzyme activity. Inactivations were roughly pseudo-first order, and t 1/2 gives the time it took to reach 50% of the starting activity in two separate experiments.

Subtilisin variant	t 1/2 (alkaline autolysis) Exp. #1	Exp. #2	t 1/2 (thermal autolysis) Exp. #1	Exp. #2
wild type	30	25	20	23
F50/V107/R213	49	41	18	23
R204	35	32	24	27
C204	43	46	38	40
C204/R213	50	52	32	36
L204/R213	32	30	20	21

G. Random Mutagenesis at Codon 204

Based on the above results, codon 204 was targeted for random mutagenesis. Mutagenic DNA cassettes (for codon at 204) all contained a fixed R213 mutation which was found to slightly augment the stability of the C204 mutant.

Plasmid DNA encoding the subtilisin mutant C204/R213 was digested with SstI and EcoRI and a 1.0 kb EcoRI/SstI fragment was isolated by electro-elution from polyacrylamide gel (fragment 1, see Fig. 35).

C204/R213 was also digested with SmaI and EcoRI and the large 4.7 kb fragment, including vector sequences and the 3' portion of coding region, was isolated from low melting agarose (fragment 2, see Fig. 36).

Fragments 1 and 2 were combined in four separate three-way ligations with heterophosphorylated fragments 3 (see Figs. 36 and 37). This heterophosphorylation of synthetic duplexes should preferentially drive the phosphorylated strand into the plasmid ligation product. Four plasmid pools, corresponding to the four ligations, were restricted with SmaI in order to linearize any single cut C204/R213 present from fragment 2 isolation, thus reducing the background of C204/R213. E. coli was then re-transformed with

SmaI-restricted plasmid pools to yield a second set of plasmid pools which are essentially free of C204/R213 and any non-segregated heterduplex material.

These second enriched plasmid pools were then used to transform B. subtilis (BG2036) and the resulting four mutant pools were screened for clones expressing subtilisin resistant to high pH/temperature inactivation. Mutants found positive by such a screen were further characterized and identified by sequencing.

The mutant L204/R213 was found to be slightly more stable than the wild type subtilisin. See Table XXII.

Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

Claims

1. A subtilisin mutant derived by the substitution of at least one amino acid residue of a precursor subtilisin with a different amino acid, so that the subtilisin mutant has at least one property which is different from the same property of the precursor subtilisin, characterised by the substitution at one or more of Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214, and Gly215 of Bacillus amyloliquefaciens subtilisin and equivalent amino acid residues in other precursor subtilisins.

2. A subtilisin mutant having an amino acid sequence derived from the amino acid sequence of a precursor subtilisin by the substitution of more than one amino acid residue of said amino acid sequence of said precursor subtilisin by a different amino acid, so that the subtilisin mutant has at least one property which is different from the same property of the precursor subtilisin, characterized by substitutions at more than one of Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214, and Gly215 of Bacillus amyloliquefaciens subtilisin and equivalent amino acid residues in other precursor subtilisins, with the proviso that when substitution is made at any residue in the group Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156 Gly166, Gly169, Phe189, Tyr217 and Met222 a substitution is also made at at least one specified position not of that group.

3. The mutant of claim 2 wherein said combinations are selected from Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Thr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 and Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217.

4. A subtilisin mutant derived by the deletion of one or more amino acid residues in a precursor subtilisin equivalent to 161-164 in B. amyloliquefaciens subtilisin, said deletion being made alone or in combination with substitutions in the amino acid sequence of the precursor subtilisin, and producing at least one property which is different from the same property of the precursor subtilisin.

5. A subtilisin mutant having altered substrate specificity when compared to a precursor subtilisin, the mutant being derived by the substitution of a different amino acid at the residue equivalent to Leu+126 of B. amyloliquefaciens subtilisin, alone or in combination with other substitutions or deletions in the amino acid sequence of the precursor subtilisin.

6. A subtilisin mutant having altered substrate specificity when compared to a precursor subtilisin, the mutant being derived by the substitution of a different amino acid at the residue equivalent to Asp+99 in B. amyloliquefaciens subtilisin, alone or in combination with other substitutions or deletions in the amino acid sequence of the precursor subtilisin.

7. A DNA sequence encoding the mutant of any one of the preceding claims.

8. An expression vector containing the mutant DNA sequence of claim 7.

9. A host cell transformed with the expression vector or claim 8.

Patentansprüche

1. Subtilisinmutante, die durch Substitution zumindest eines Aminosäurerests eines Vorläufer-Subtilisins durch eine davon verschiedene Aminosäure hergeleitet ist, sodaß die Subtilisinmutante zumindest eine Eigenschaft aufweist, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet, gekennzeichnet durch die Substitution an einem oder mehreren von Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 und Gly215 von Bacillus amyloliquefaciens-Subtilisin und äquivalenten Aminosäureresten in anderen Vorläufer-Subtilisinen.

2. Subtilisinmutante mit einer Aminosäuresequenz, die aus der Aminosäuresequenz eines Vorläufer-Subtilisins durch Substitution mehr als eines Aminosäurerests der Aminosäuresequenz des Vorläufer-Subtilisins durch eine davon verschiedene Aminosäure hergeleitet ist, sodaß die Subtilisinmutante zumindest eine Eigenschaft auWeist, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet, gekennzeichnet durch Substitutionen an mehr als einem von Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 und Gly215 von Bacillus amyloliquefaciens-Subtilisin und äquivalenten Aminosäureresten in anderen Vorläufer-Subtilisinen, mit der Maßgabe, daß bei einer Substitution an irgendeinem Rest in der Gruppe Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217 und Met222 eine Substitution auch an zumindest einer bestimmten Position durchgeführt wird, die nicht dieser Gruppe angehört.

3. Mutante nach Anspruch 2, worin die Kombinationen aus Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Tyr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 und Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217 ausgewählt sind.

4. Subtilisinmutante, die durch Löschung eines oder mehrerer Aminosäurereste in einem Vorläufer-Subtilisin, das 161-164 in B. amyloliquefaciens-Subtilisin äquivalent ist, hergeleitet ist, wobei die Löschung entweder alleine oder in Kombination mit Substitutionen in der Aminosäuresequenz des Vorläufer-Subtilisins erfolgt, und zumindest eine Eigenschaft ergibt, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet.

5. Subtilisinmutante mit geänderter Substratspezifität im Vergleich zu einem Vorläufersubtilisin, wobei die Mutante durch Substitution einer unterschiedlichen Aminosäure am Rest, der Leu + 126 von B. amyloliquefaciens-Subtilisin äquivalent ist, alleine oder in Kombination mit anderen Substitutionen oder Löschungen in der Aminosäuresequenz des Vorläufer-Subtilisins hergeleitet ist.

6. Subtilisinmutante mit geänderter Substratspezifität im Vergleich zu einem Vorläufersubtilisin, wobei die Mutante durch Substitution einer unterschiedlichen Aminosäure am Rest, der Asp +99 im B. amyloliquefaciens-Subtilisin äquivalent ist, alleine oder in Kombination mit anderen Substitutionen oder Löschungen in der Aminosäuresequenz des Vorläufer-Subtilisins hergeleitet ist.

7. DNA-Sequenz, die für die Mutante nach einem der vorhergehenden Ansprüche kodiert.

8. Expressionsvektor, der die Mutanten-DNA-Sequenz von Anspruch 7 enthält.

9. Wirtszelle, die mit dem Expressionsvektor von Anspruch 8 transformiert ist.

Revendications

1. Mutant de subtilisine dérivé par la substitution d'au moins un résidu d'acide aminé d'une subtilisine précurseur et par un acide aminé différent de manière que le mutant de subtilisine ait au moins une propriété qui est différente de la même propriété de la subtilisine précurseur, caractérisé par la substitution à un ou plusieurs de Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 et Gly215 de la subtilise de Bacillus amyloliquefaciens et les résidus d'acides aminés équivalents dans d'autres subtilisines précurseurs.

2. Mutant de subtilisine ayant une séquence d'acides aminés dérivée de la séquence d'acides aminés d'une subtilisine précurseur par la substitution de plus d'un résidu d'acide aminé de ladite séquence d'acides aminés de ladite subtilisine précurseur par un acide aminé différent de manière que le mutant de subtilisine ait au moins une propriété qui est différente de la même propriété de la subtilisine précurseur, caractérisé par des substitutions à plus d'un de Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 et Gly215 de la subtilisine de Bacillus amyloliquefaciens et des résidus d'acides aminés équivalents dans d'autres subtilisines précurseurs, à condition que quand la substitution est effectuée à tout résidu dans le groupe formé de Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217 et Met222, une substitution soit également effectuée en au moins une position spécifiée ne faisant pas partie de ce groupe.

3. Mutant de la revendication 2 où lesdites associations sont choisies parmi Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Thr22/ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 et Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217.

4. Mutant de subtilisine dérivé par la délétion d'un ou plusieurs résidus d'acides aminés dans une subtilisine précurseur équivalente à 161-164 dans la subtilisine de B. amyloliquefaciens, ladite délétion étant effectuée seule ou en association avec des substitutions dans la séquence d'acides aminés de la subtilisine précurseur et la production d'au moins une propriété qui est différente de la même propriété de la subtilisine précurseur.

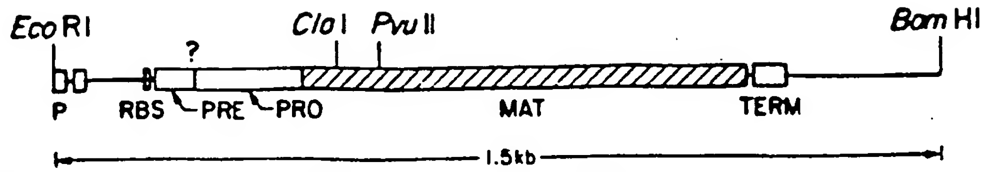
5. Mutant de subtilisine ayant une spécificité modifiée du substrat en comparaison avec une subtilisine précurseur, le mutant étant dérivé par la substitution d'un acide aminé différent au résidu équivalent à Leu + 126 de la subtilisine de B. amyloliquefaciens, seule ou en association avec d'autres substitutions ou délétions dans la séquence d'acides aminés de la subtilisine précurseur.

6. Mutant de subtilisine ayant une spécificité modifiée de substrat en comparaison avec une subtilisine précurseur, le mutant étant dérivé par la substitution d'un acide aminé différent au résidu équivalent à Asp + 99 dans la substilisine de B. amyloliquefaciens, seule ou en association avec d'autres substitutions ou délétions dans la séquence d'acides aminés de la subtilisine précurseur.

7. Séquence d'ADN codant le mutant selon l'une quelconque des revendications précédentes.

8. Vecteur d'expression contenant la séquence d'ADN du mutant de la revendication 7.

9. Cellule hôte transformée par le vecteur d'expression de la revendication.8 .



(5) (3) (4)

P RBS -107 fMet

1 GGTCTACTAAAATATTATTCCATACTATACAATTAATACACAGAATAATCTGTCTATTGGTTATTCTGCAAATGAAAAAAAGGAGAGGATAAAGA GTG

-100 PRE -90

Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser
99 AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA TCC

-80 -70 PRO -60

Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
174 TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG

-50 -40

Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala
249 AGC GCC GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA

-30 -20 -10

Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp
324 GCT TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT

-1 1 MAT 10

His Val Ala His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln
399 CAC GTA GCA CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA

20 30 40

Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asn Leu Lys Val
474 GGC TAC ACT GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT CCT GAT TTA AAG GTA

50 Pro Asn 60 Asp

Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His Gly Thr His Val Ala
549 GCA GGC GGA GCC AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC AAC TCT CAC GGA ACT CAC GTT GCC

70 80 Ser Ala 90

Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys
624 GGC ACA GTT GCG GCT CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA AAA

Asp Ala 100 110

Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met
699 GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT AAC GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG

120 130 140

Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
774 GAC GTT ATT AAC ATG AGC CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT GAT AAA GCC GTT GCA

150 Ser Thr 160

Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val Gly Tyr Pro Gly
849 TCC GGC GTC GTA GTC GTT GCG GCA GCC GGT AAC GAA GGC ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT

170 180 190

Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly Pro
924 AAA TAC CCT TCT GTC ATT GCA GTA GGC GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC GTA GGA CCT

200 210

Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly
999 GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT

220 230 240

Thr Ser Met Ala Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn Thr
1074 ACG TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC ACT

250 Gln 260

Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn
1149 CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC

270 275 TERM

Val Gln Ala Ala Ala Gln OC
1224 GTA CAG GCG GCA GCT CAG TAA AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTATTTTTCTTCCTCCGCATGTTCAATCCGCTCC

1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCG

1416 CTTCCCGGTTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGCGTTTTCCTGATACCGGGAGACGGCATTCGTAATCGGATC

FIG.—1

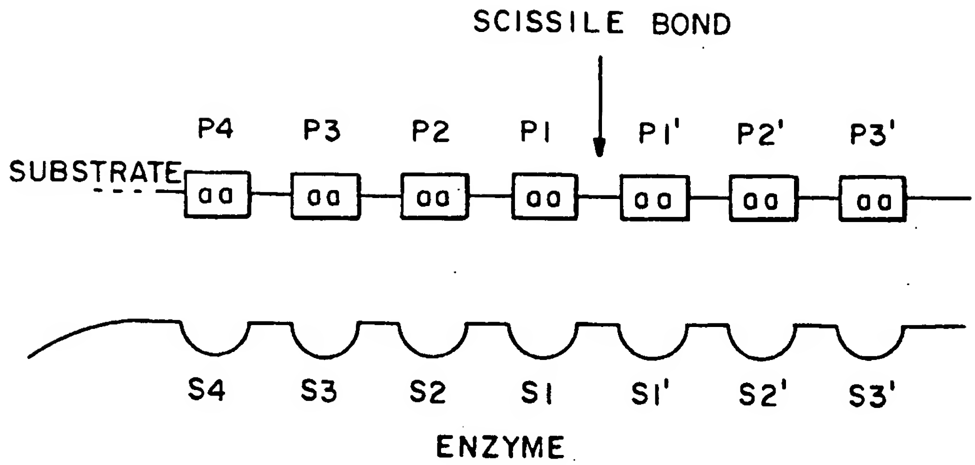

SCISSILE BOND
P4
P3
P2
P1
P1'
P2'
P3'
SUBSTRATE
aa
aa
aa
aa
aa
aa
aa
S4
S3
S2
S1
S1'
S2'
S3'
ENZYME

FIG. -2

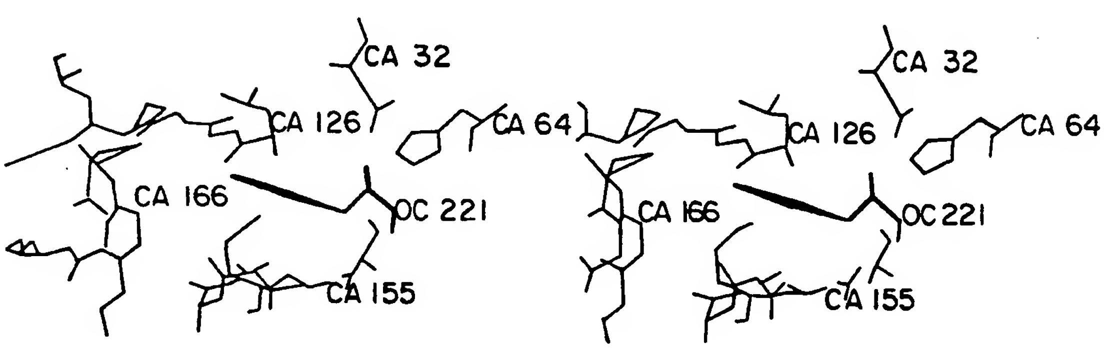

CA 32
CA 126
CA 64
CA 166
OC 221
CA 155
CA 32
CA 126
CA 64
CA 166
OC 221
CA 155

FIG. — 3

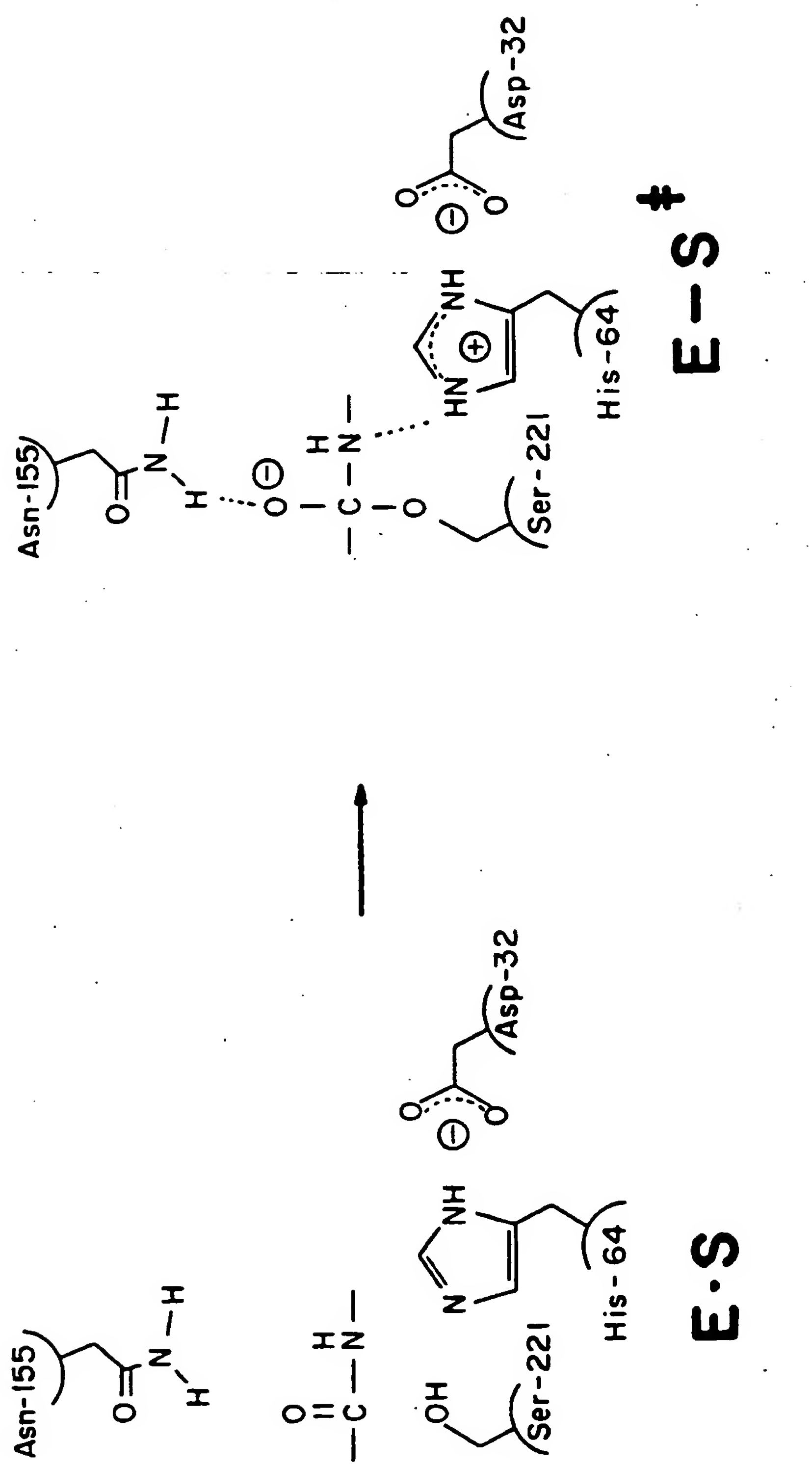


FIG. — 4

Homology of Bacillus proteases

1. Bacillus amyloliquifaciens
2. Bacillus subtilis var.I168
3. Bacillus licheniformis (carlsbergensis)

1									10										20
A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A	L	H	S	Q	G
A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P	A	L	H	S	Q	G
A	Q	T	V	P	Y	G	I	P	L	I	K	A	D	K	V	Q	A	Q	G

21									30										40
Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P
Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P
F	K	G	A	N	V	K	V	A	V	L	D	T	G	I	Q	A	S	H	P

41									50										60
D	L	K	V	A	G	G	A	S	M	V	P	S	E	T	N	P	F	Q	D
D	L	N	V	R	G	G	A	S	F	V	P	S	E	T	N	P	Y	Q	D
D	L	N	V	V	G	G	A	S	F	V	A	G	E	A	Y	N	T	•	D

61									70										80
N	N	S	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I	G
G	S	S	H	G	T	H	V	A	G	T	I	A	A	L	N	N	S	I	G
G	N	G	H	G	T	H	V	A	G	T	V	A	A	L	D	N	T	T	G

81									90										100
V	L	G	V	A	P	S	A	S	L	Y	A	V	K	V	L	G	A	D	G
V	L	G	V	S	P	S	A	S	L	Y	A	V	K	V	L	D	S	T	G
V	L	G	V	A	P	S	V	S	L	Y	A	V	K	V	L	N	S	S	G

101									110										120
S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	N	M	D
S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	S	N	N	M	D
S	G	S	Y	S	G	I	V	S	G	I	E	W	A	T	T	N	G	M	D

FIG.—5A-I

121									130										140
V	I	N	M	S	L	G	G	P	S	G	S	A	A	L	K	A	A	V	D
V	I	N	M	S	L	G	G	P	T	G	S	T	A	L	K	T	V	V	D
V	I	N	M	S	L	G	G	A	S	G	S	T	A	M	K	Q	A	V	D

141									150										160
K	A	V	A	S	G	V	V	V	V	A	A	A	G	N	E	G	T	S	G
K	A	V	S	S	G	I	V	V	A	A	A	A	G	N	E	G	S	S	G
N	A	Y	A	R	G	V	V	V	V	A	A	A	G	N	S	G	N	S	G

161									170										180
S	S	S	T	V	G	Y	P	G	K	Y	P	S	V	I	A	V	G	A	V
S	T	S	T	V	G	Y	P	A	K	Y	P	S	T	I	A	V	G	A	V
S	T	N	T	I	G	Y	P	A	K	Y	D	S	V	I	A	V	G	A	V

181									190										200
D	S	S	N	Q	R	A	S	F	S	S	V	G	P	E	L	D	V	M	A
N	S	S	N	Q	R	A	S	F	S	S	A	G	S	E	L	D	V	M	A
D	S	N	S	N	R	A	S	F	S	S	V	G	A	E	L	E	V	M	A

201									210										220
P	G	V	S	I	Q	S	T	L	P	G	N	K	Y	G	A	Y	N	G	T
P	G	V	S	I	Q	S	T	L	P	G	G	T	Y	G	A	Y	N	G	T
P	G	A	G	V	Y	S	T	Y	P	T	N	T	Y	A	T	L	N	G	T

221									230										240
S	M	A	S	P	H	V	A	G	A	A	A	L	I	L	S	K	H	P	N
S	M	A	T	P	H	V	A	G	A	A	A	L	I	L	S	K	H	P	T
S	M	A	S	P	H	V	A	G	A	A	A	L	I	L	S	K	H	P	N

241									250										260
W	T	N	T	Q	V	R	S	S	L	E	N	T	T	T	K	L	G	D	S
W	T	N	A	Q	V	R	D	R	L	E	S	T	A	T	Y	L	G	N	S
L	S	A	S	Q	V	R	N	R	L	S	S	T	A	T	Y	L	G	S	S

261									270					
F	Y	Y	G	K	G	L	I	N	V	Q	A	A	A	Q
F	Y	Y	G	K	G	L	I	N	V	Q	A	A	A	Q
F	Y	Y	G	K	G	L	I	N	V	E	A	A	A	Q

FIG.—5A-2

ALIGNMENT OF B.AMYLOLIQUIFACIENS SUBTILISIN AND THERMITASE
1.B.amyloliquifaciens subtilisin
2.thermitase

1																10			
A	Q	S	V	•	P	Y	•	•	•	•	•	•	G	V	S	Q	I	K	A
Y	T	P	N	D	P	Y	F	S	S	R	Q	Y	G	P	Q	K	I	Q	A

						20										30			
P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S
P	Q	A	W	D	I	A	E	•	G	S	G	A	K	I	A	I	V	D	T

						40												50	
G	I	D	S	S	H	P	D	L	•	•	K	V	A	G	G	A	S	M	V
G	V	Q	S	N	H	P	D	L	A	G	K	V	V	G	G	W	D	F	V

								60										70	
P	S	E	T	N	P	F	Q	D	N	N	S	H	G	T	H	V	A	G	T
D	N	D	S	T	P	•	Q	N	G	N	G	H	G	T	H	C	A	G	I

									80										90
V	A	A	L	•	N	N	S	I	G	V	L	G	V	A	P	S	A	S	L
A	A	A	V	T	N	N	S	T	G	I	A	G	T	A	P	K	A	S	I

									100										110
Y	A	V	K	V	L	G	A	D	G	S	G	Q	Y	S	W	I	I	N	G
L	A	V	R	V	L	D	N	S	G	S	G	T	W	T	A	V	A	N	G

									120										130
I	E	W	A	I	A	N	N	M	D	V	I	N	M	S	L	G	G	P	S
I	T	Y	A	A	D	Q	G	A	K	V	I	S	L	S	L	G	G	T	V

									140										150
G	S	A	A	L	K	A	A	V	D	K	A	V	A	S	G	V	V	V	V
G	N	S	G	L	Q	Q	A	V	N	Y	A	W	N	K	G	S	V	V	V

FIG.—5B—1

```
                                     160                                   170
A  A  A  G  N  E  G  T  S  G  S  S  S  T  V  G  Y  P  G  K
A  A  A  G  N  A  G  N  T  A  •  •  •  •  P  N  Y  P  A  Y

                                     180                                   190
Y  P  S  V  I  A  V  G  A  V  D  S  S  N  Q  R  A  S  F  S
Y  S  N  A  I  A  V  A  S  T  D  Q  N  D  N  K  S  S  F  S

                                     200                                   210
S  V  G  P  E  L  D  V  M  A  P  G  V  S  I  Q  S  T  L  P
T  Y  G  S  V  V  D  V  A  A  P  G  S  W  I  Y  S  T  Y  P

                                     220                                   230
G  N  K  Y  G  A  Y  N  G  T  S  M  A  S  P  H  V  A  G  A
T  S  T  Y  A  S  L  S  G  T  S  M  A  T  P  H  V  A  G  V

                                     240                                   250
A  A  L  I  L  S  K  H  P  N  W  T  N  T  Q  V  R  S  S  L
A  G  L  L  A  S  Q  G  R  S  •  •  A  S  N  I  R  A  A  I

                                        260
E  N  T  T  T  K  •  L  G  D  S  F  Y  Y  G  K  G  L  I  N
E  N  T  A  D  K  I  S  G  T  G  T  Y  W  A  K  G  R  V  N

270
V  Q  A  A  A  Q
A  Y  K  A  V  Q  Y
```

FIG.—5B-2

TOTALLY CONSERVED RESIDUES IN SUBTILISINS

```
1                  10                  20
. . . . P . . . . . . . . . . . . . . .

21                 30                  40
. . G . . . . . . . . D . G . . . . H .

41                 50                  60
. . . . . G . . . . V . . . . . . . . .

61                 70                  80
. . . H G T H . . G . . . . . . . . . .

81                 90                  100
. . G . . . . . . . . . . . V L . . . G

101                110                 120
S G . . . . . . . G . . . . . . . . . .

121                130                 140
. . . . . L G . . . . . . . . . . . . .

141                150                 160
. . . . . G . . . . . . . G N . . . . .

161                170                 180
. . . . . . Y P . . . . . . . . V . . .

181                190                 200
. . . . . . . S F S . . . . . . . . . .

201                210                 220
P G . . . . . . . . . . . . . . . . G T

221                230                 240
S M A . P H V A G . . . . . . . . . . .

241                250                 260
. . . . . . R . . . . . . . . . . . . .

261                270
. . . . . . . . N . . . . . .
```

FIG.—5C

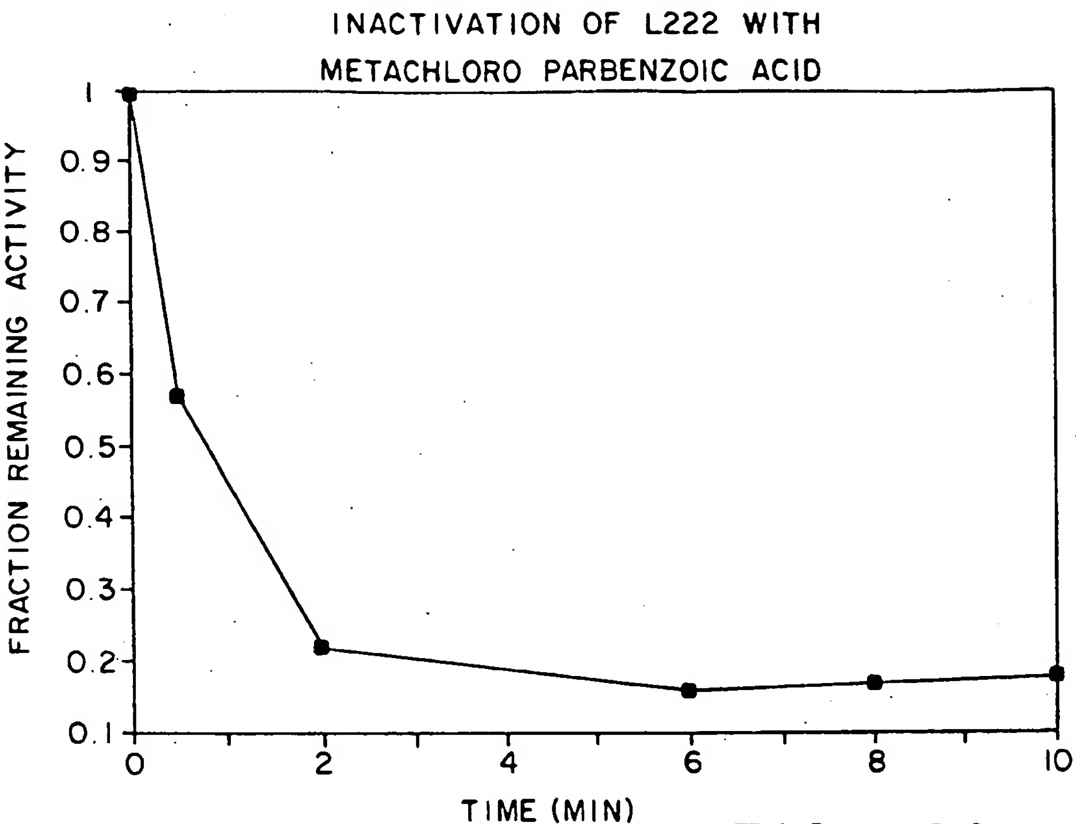

INACTIVATION OF L222 WITH
METACHLORO PARBENZOIC ACID
FRACTION REMAINING ACTIVITY
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
TIME (MIN)

FIG. — 6A

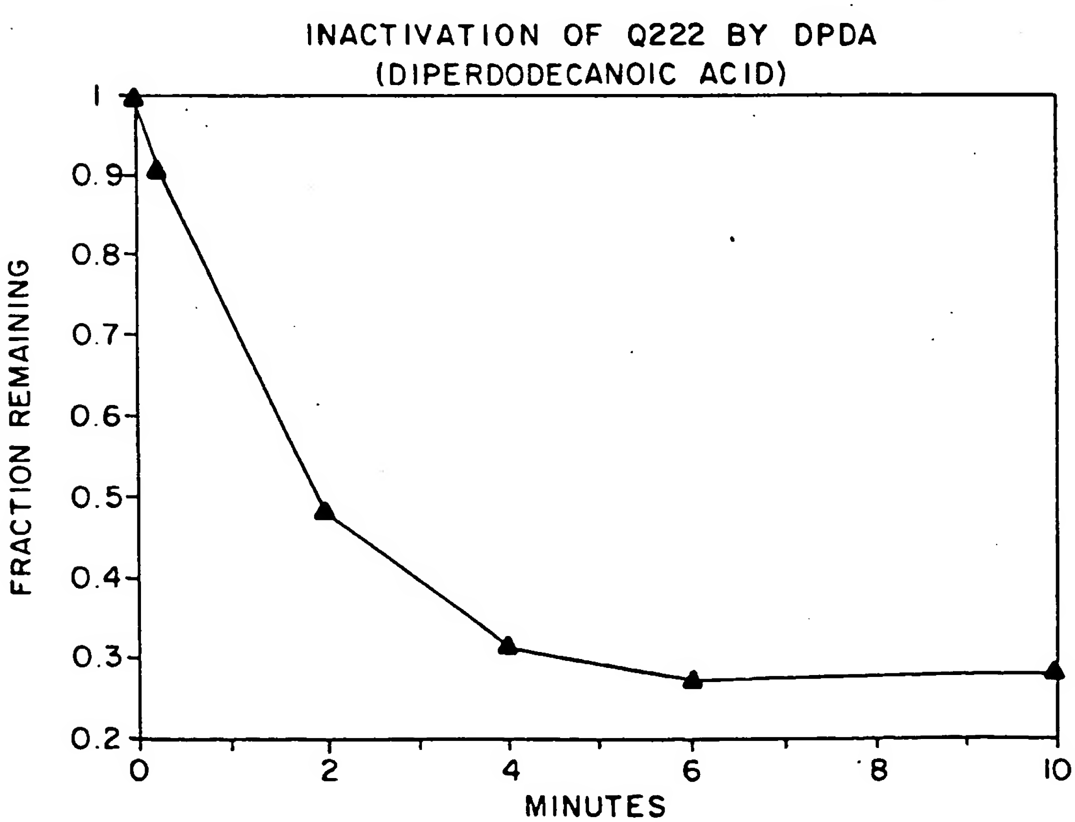

INACTIVATION OF Q222 BY DPDA
(DIPERDODECANOIC ACID)
FRACTION REMAINING
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
10
MINUTES

FIG. — 6B

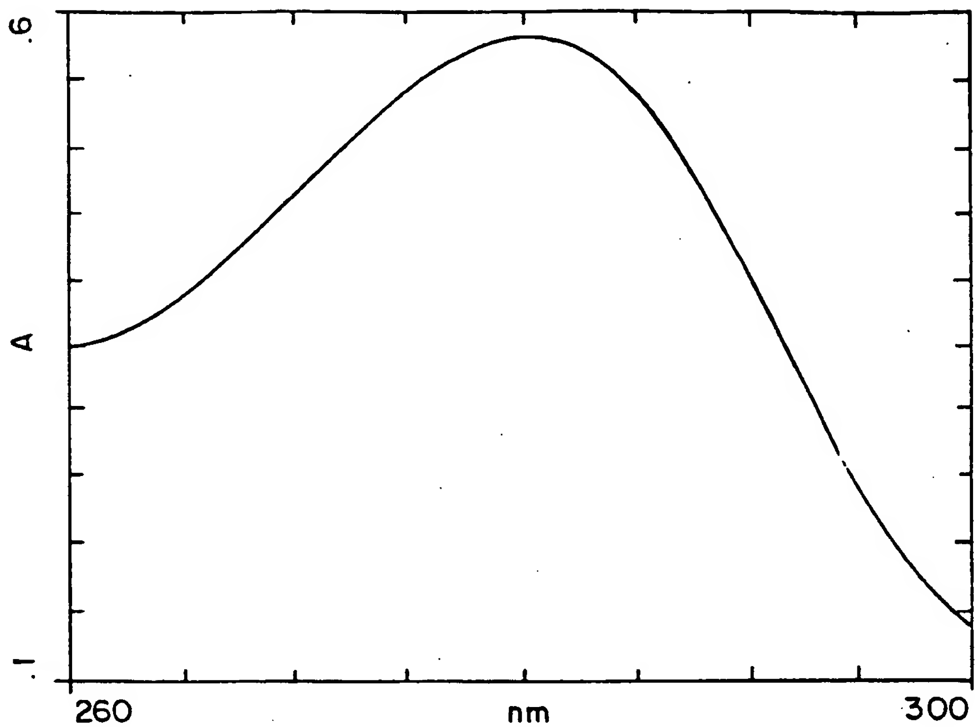


FIG. —7A

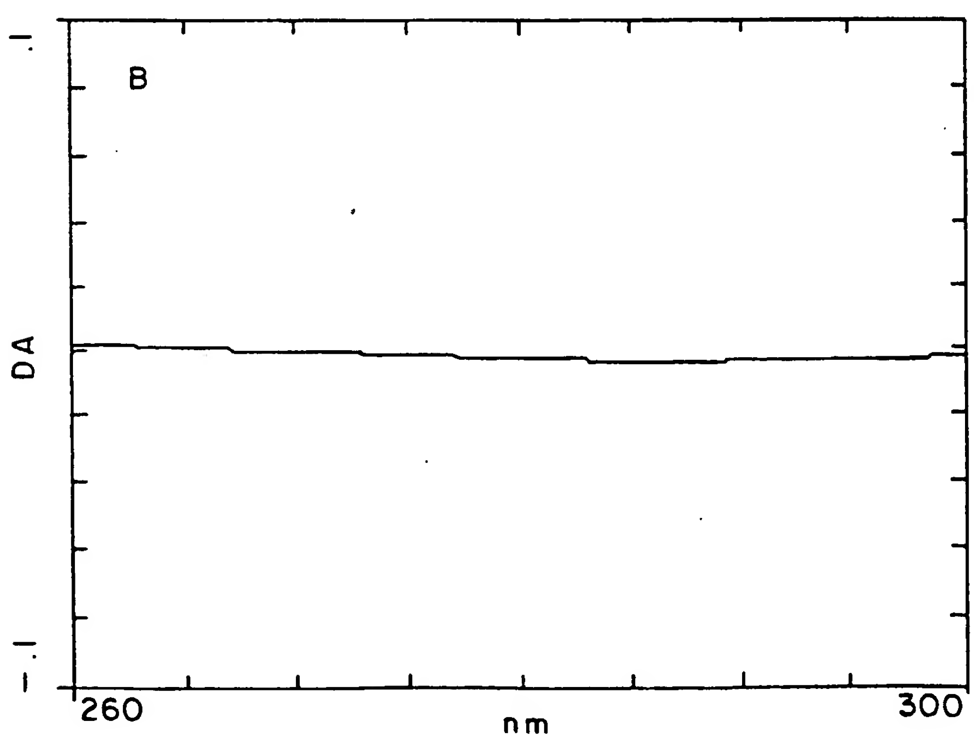


FIG. — 7B

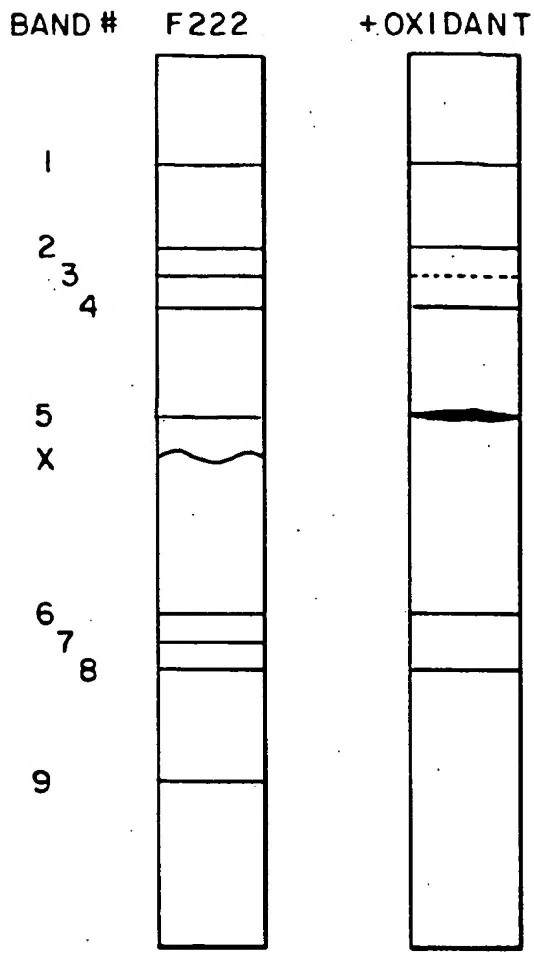

BAND #
F222
+OXIDANT
1
2
3
4
5
X
6
7
8
9

FIG.— 8

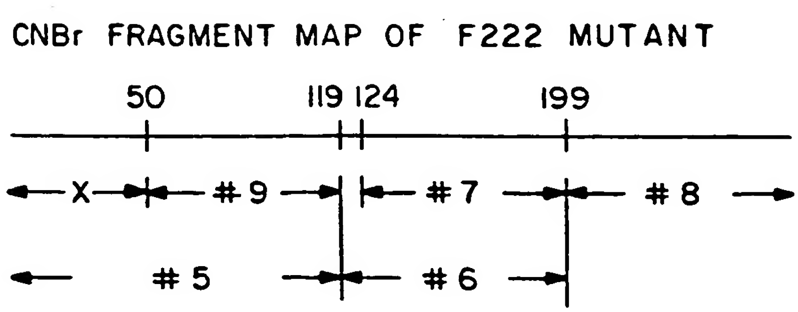

CNBr FRAGMENT MAP OF F222 MUTANT
50
119
124
199
X
9
7
8
5
6

FIG.—9

```
1. Codon number:                          43      45
2. Wild type amino acid sequence:         Lys-Val-Ala-Gly-Gly-Ala-Ser-Met-Val-Pro-Ser
3. Wild type DNA sequence:              5'-AAG-GTA-GCA-GGC-GGA-GCC-AGC-ATG-GTT-CCT-TCT
                                           TTC-CAT-CGT-CCG-CCT-CGG-TCG-TAC-CAA-GGA-AGA-5'

                                               ** *                         *
4. pΔ50:                                5'-AAG-GCC-T----------------GC-ATG-GTA-CCT-TCT
                                           TTC-CGG-A----------------CG-TAC-CAT-GGA-AGA-5'
                                              Stu I                         Kpn I

                                                                            *
5. pΔ50 cut with Stu I/Kpn I            5'-AAG-G                              pCT-TCT
                                           TTC-Cp                         CAT-GGA-AGA-5'

                                                                            *
6. Cut pΔ50 ligated with cassettes:     5'-AAG-GTA-GCA-GGC-GGA-GCC-AGC-ATG-GTA-CCT-TCT
                                           TCC-CAT-CGT-CCG-CCT-CGG-TCG-TAC-CAT-GGA-AGA-5'

                                                          ***         *
7. Mutagenesis primer for pΔ50:         5'-CT-GAT-TTA-AAG-GCC-TGC-ATG-GTA-CCT-TCT-GA

8. Mutants made:                        V45, P45, V45/P48, E46, E48, V48, C49, C50, F50
```

FIG.—10

```
1. Codon number:                      117        120             124   126             130
2. Wild type amino acid sequence:  Asn-Asn-Met-Asp-Val-Ile-Asn-Met-Ser-Leu-Gly-Gly-Pro-Ser
3. Wild type DNA sequence:      5'-AAC-AAT-ATG-GAC-GTT-ATT-AAC-ATG-AGC-CTC-GGC-GGA-CCT-TCT
                                   TTG-TTA-TAC-CTG-CAA-TAA-TTG-TAC-TCG-GAG-CCG-CCT-GGA-AGA-5'

                                               * ***                       *   *
4. pΔ124:                       5'-AAC-AAT-ATG-GAT-ATC-------------------C-GGG-GGC-CCT-TCT
                                   TTG-TTA-TAC-CTA-TAG-------------------G-CCC-CCG-GGA-AGA-5'
                                               Eco RV                          Apa I

                                               *                               *
5. pΔ124 cut with Eco RV        5'-AAC-AAT-ATG-GAT                                 pCT-TCT
   and Apa I                       TTG-TTA-TAC-CTAp                            CCG-GGA-AGA-5'

                                               *                                   *
6. Cut pΔ124 ligated with       5'-AAC-AAT-ATG-GAT-GTT-ATT-AAC-ATG-AGC-CTC-GGC-GGC-CCT-TCT
   cassettes:                      TTG-TTA-TAC-CTA-CAA-TAA-TTG-TAC-TCG-GAG-CCG-CCG-GGA-AGA-5'

                                               * ***       *   *
7. Mutagenesis primer           5'-AAC-AAT-ATG-GAT-ATC-C-GGG-GGC-CCT-TCT-GGT-TC-3'
   for pΔ124::

8. Mutants made:                I 124, L 124 AND C126
```

FIG.—11

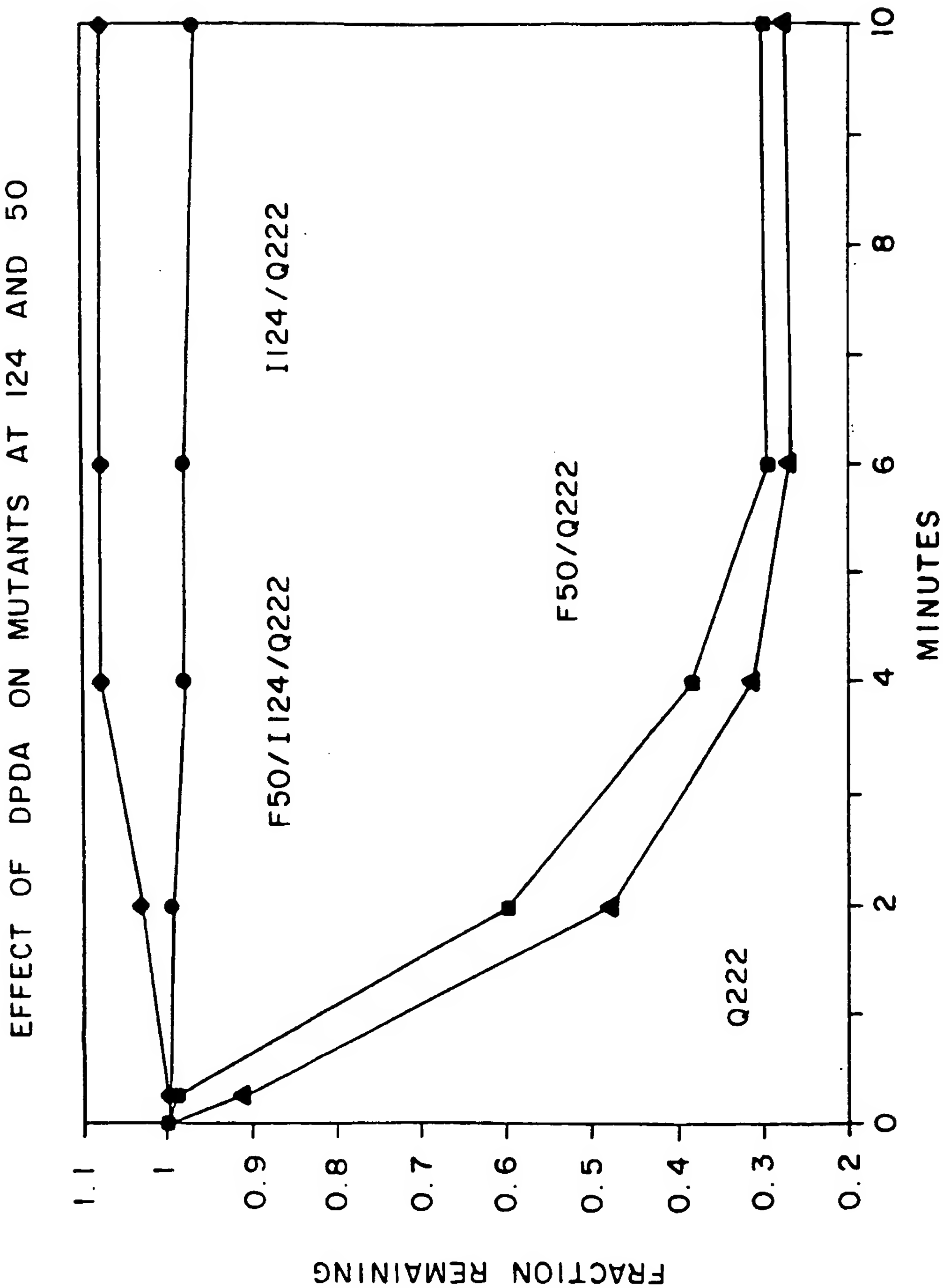

EFFECT OF DPDA ON MUTANTS AT 124 AND 50
I124/Q222
F50/I124/Q222
F50/Q222
Q222
FRACTION REMAINING
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
10
MINUTES

FIG.-12

```
                              Codon:                               166
Wild type amino acid sequence:        Thr Ser Gly Ser Ser Ser Thr Val Gly Tyr Pro Gly

1.  Wild type DNA sequence:          5'-ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT-3'
                                     3'-TGA AGG CCG TCG AGT TCG TGT CAC CCG ATG GGA CCA-5'

                                                *                             *
2.  pΔ166 DNA sequence:              5'-ACT TCC GGG AGC TCA A------------- C CCG GGT-3'
                                     3'-TGA AGG CCC TCG AGT T              G GGC CCA-5'
                                                    SacI                      XmaI

                                                *                             *
3.  pΔ166 cut with SacI and XmaI:    5'-ACT TCC GGG AGC T                  pCCG GGT-3'
                                     3'-TGA AGG CCCp                              CA-5'

                                                *                  ***        *
4.  Cut pΔ166 ligated with           5'-ACT TCC GGG AGC TCA AGC ACA GTG NNN TAC CCG GGT-3'
    duplex DNA cassette pools:       3'-TGA AGG CCC TCG AGT TCG TGT CAC NNN ATG GGC CCA-5'
```

MUTAGENESIS PRIMER 37 MER

5' AA GGC ACT TCC GGG AGC TCA ACC CGG GTA AA TAC CCT 3'

FIG.—13

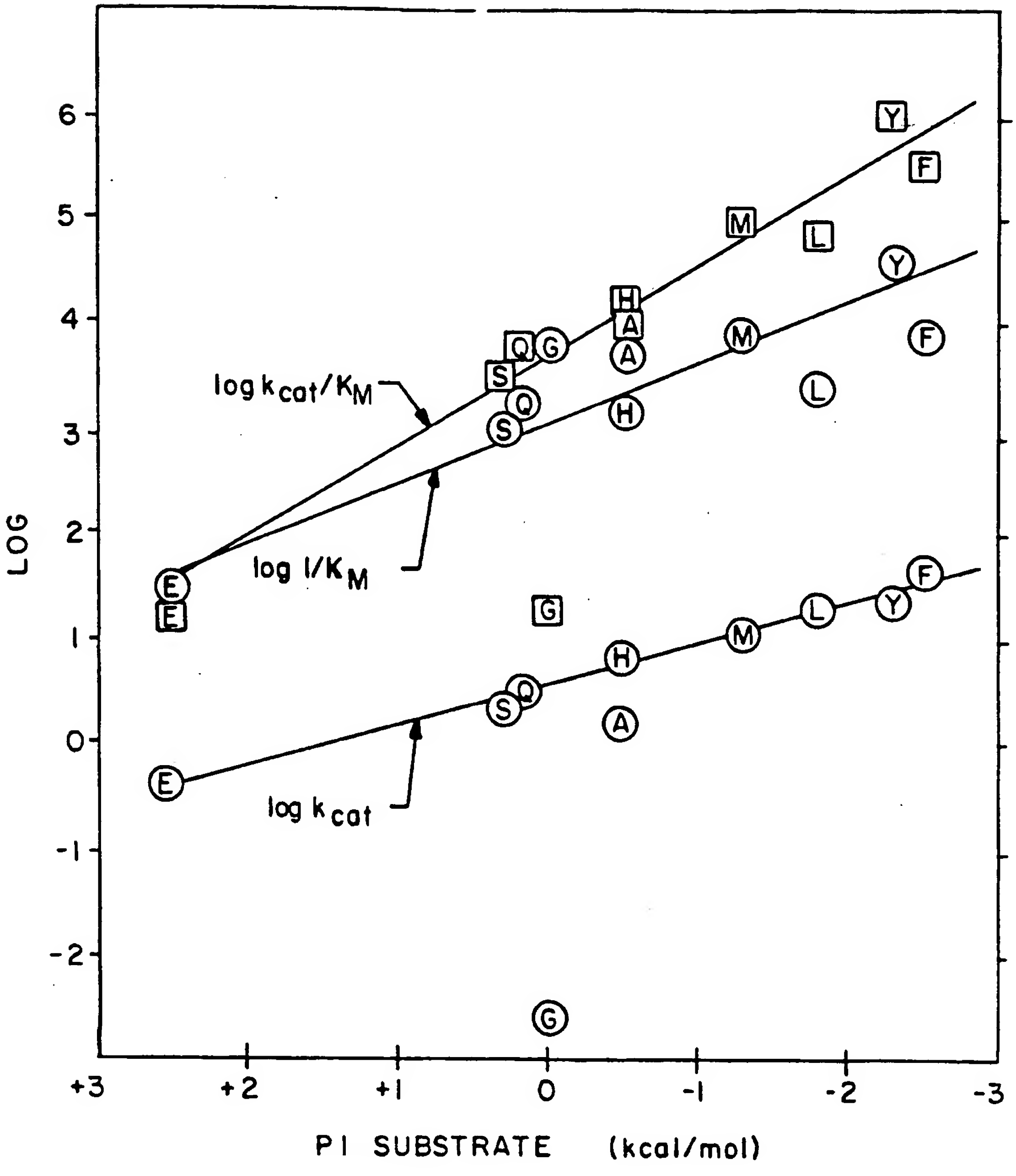

6
5
4
3
2
1
0
-1
-2
LOG
log k_{cat}/K_M
log $1/K_M$
log k_{cat}
Y
F
M
L
H
A
Q
G
S
E
+3
+2
+1
0
-1
-2
-3
P1 SUBSTRATE (kcal/mol)

FIG.-14

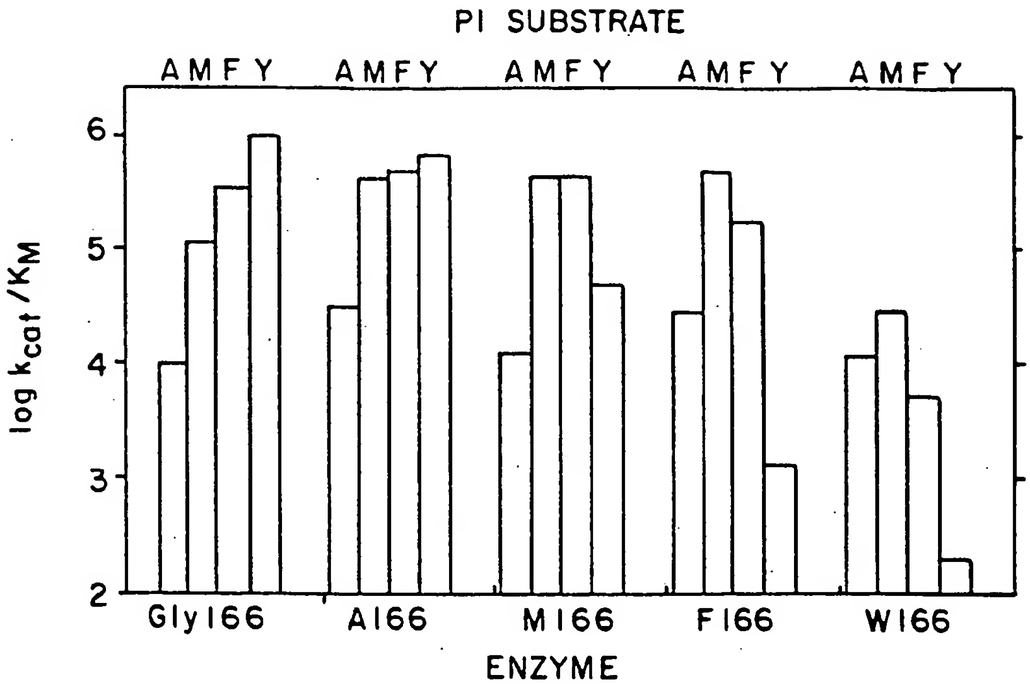


FIG. - 15A

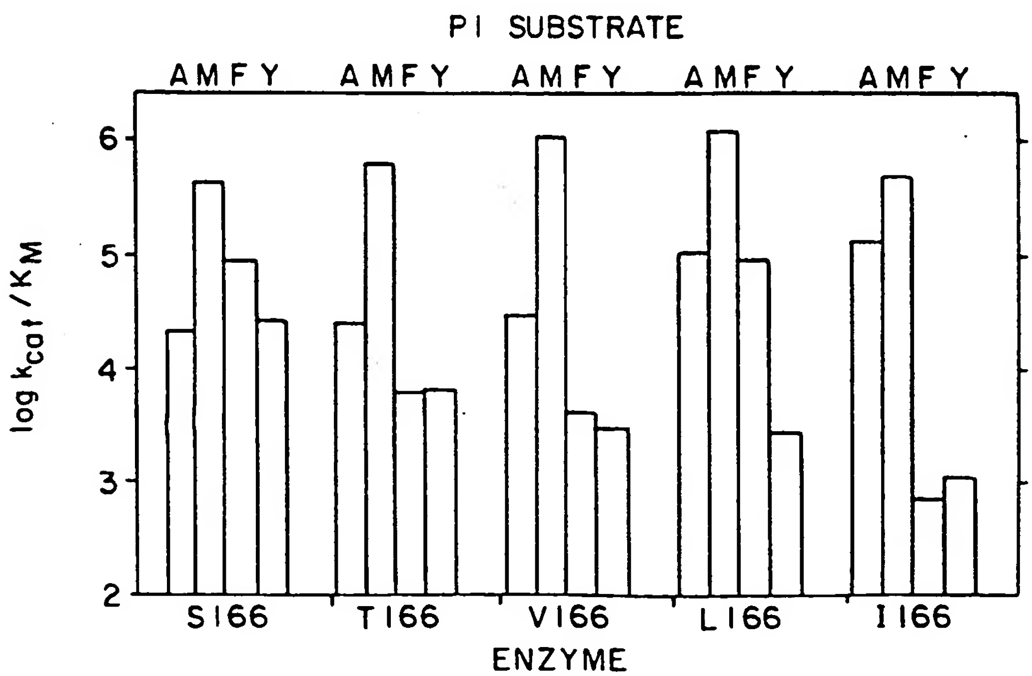


FIG. - 15B

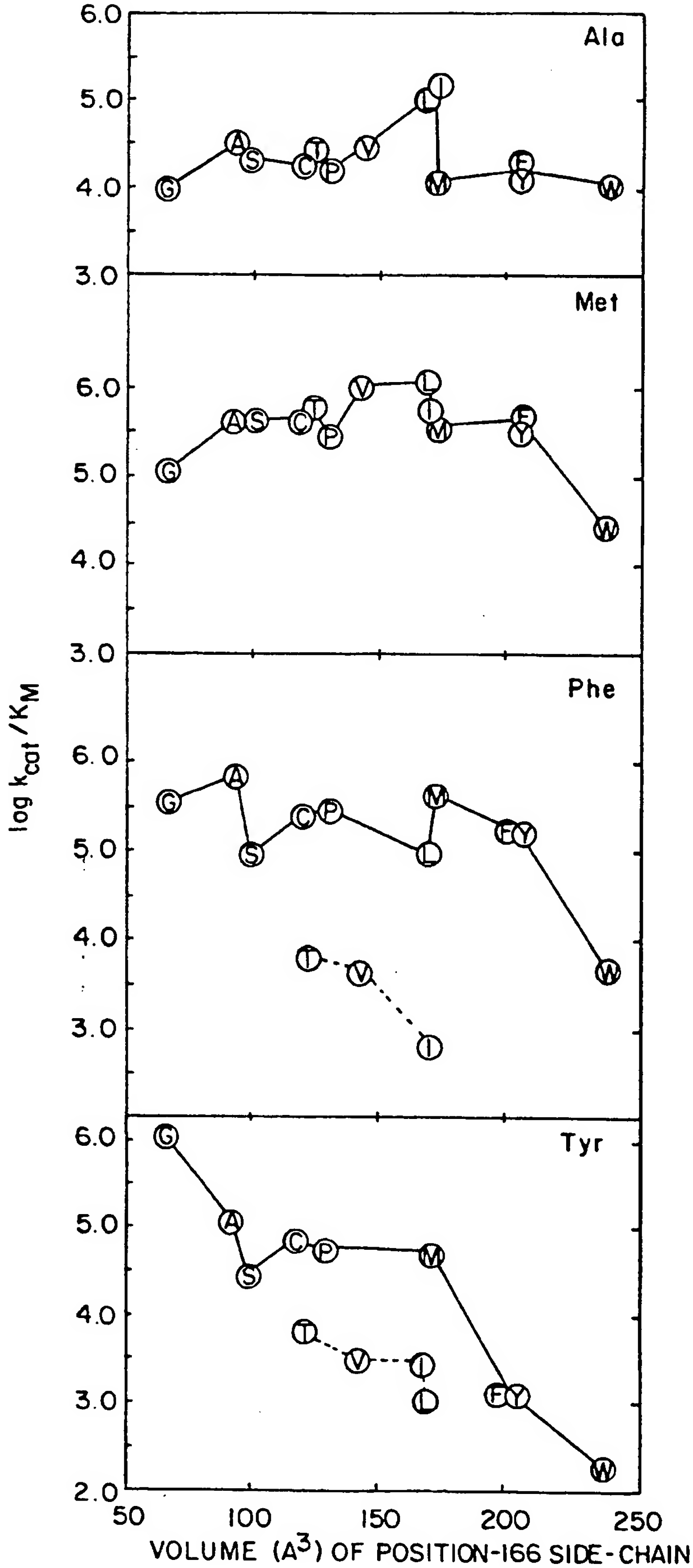

Ala
Met
Phe
Tyr
log k_{cat}/K_M
6.0
5.0
4.0
3.0
2.0
50
100
150
200
250
VOLUME (A^3) OF POSITION-166 SIDE-CHAIN
G
A
S
C
T
P
V
L
I
M
F
Y
W

FIG.-16

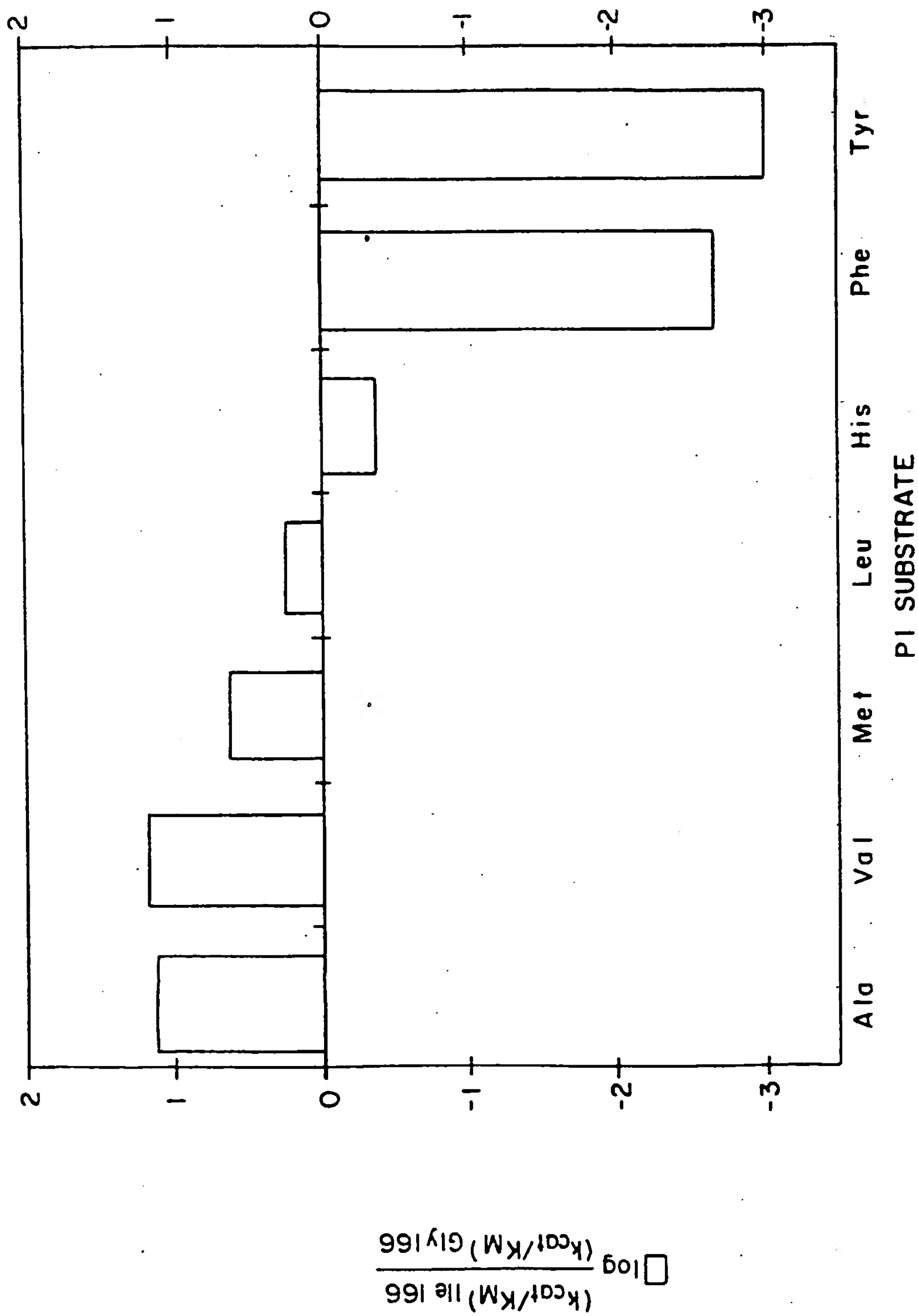

2
1
0
-1
-2
-3
Ala
Val
Met
Leu
His
Phe
Tyr
P1 SUBSTRATE
log $\frac{(k_{cat}/KM)\ Ile\ 166}{(k_{cat}/KM)\ Gly166}$

FIG. – 17

GLY-169 CASSETTE MUTAGENESIS

CODON:		162 ... 169 ... 173	
WILD TYPE AMINO ACID SEQUENCE:		SER SER THR VAL GLY TYR PRO GLY LIS TYR PRO SER	
1. WILD TYPE DNA SEQUENCE	5'	TCA AGC ACA GTG GGC TAC CCT GGT AAA TAC CCT TCT	3'
	3'	AGT TCG TGT CAC CCG ATG GGA CCA TTT ATG GGA AGA	5'
2. P169 DNA SEQUENCE	5'	TCA AGC ACA GTC GGG TAC CCT-----GA TAT CCT TCT	3'
	3'	AGT TCG TGT CAC CCC ATG GGA CT ATA GGA AGA	5'
		KPNI ECORV	
3. P169 CUT WITH KPNI AND ECORV:	5'	TAC AGC ACA GTC GGG TAC PAT CCT TCT	3'
	3'	AGT TCG TGT CAC CCP TA GGA AGA	5'
4. CUT P169 LIGATED WITH	5'	TAC AGC ACA GTG GGG TAC CCT NNN AAA TAT CCT TGT	3'
OLIGONUCLEOTIDE POOLS	3'	AGT TCG TGT CAC CCC ATG GGA NNN TTT ATA GGA AGA	5'
MUTAGENESIS PRIMER FOR P169	5'	AAG CAC AGT GGG GTA CCC TGA TAT CCT TCT GTC A	3'

FIG.—18

1. Codon number: 100 ... 104 105 ... 108
2. Wild type amino acid sequence: Gly-Ser-Gly-Gln-Tyr-Ser-Trp-Ile-Ile-
3. Wild type DNA sequence: 5'-GGT-TCC-GGC-CAA-TA$\boxed{\text{C-AGC-TG}}$G-ATC-ATT-3'
   Pvu II
4. Primer for *Hind* III insertion at 104: 5'-GGT-TCC-GGC-C$\boxed{\text{AA-GCTT}}$-AGC-TGG-ATC-ATT-3'
   ****
   Hind III
5. Primers for 104 mutants: 5'---T-TCC-GCC-CAA-NNN-AGC-TGG-ATC------3'
   ***
6. Mutants made: A, M, L, S, AND H104

FIG.—19

```
1. Codon number:                      148     150     152         155
2. Wild type amino acid sequence:   Val-Val-Val-Ala-Ala-Ala-Gly-Asn-Glu
3. Wild type DNA sequence:        5'-GTA-GTC-GTT-GCG-GCA-GCC-GGT-AAC-GAA-3'

                                                    *   *
4. V152/P153                      5'-GTA-GTC-GTT-GCG-GTA-CCC-GGT-AAC-GAA-3'
                                                    Kpn I

                                                   ***
5. S 152:                         5'-GTA-GTC-GTT-GCG-AGC-GCC-GGT-AAC-GAA-3'

                                                   **
6. G 152:                         5'-GTA-GTC-GTT-GCG-GGC-GCC-GGT-AAC-GAA-3'
```

FIG.—20

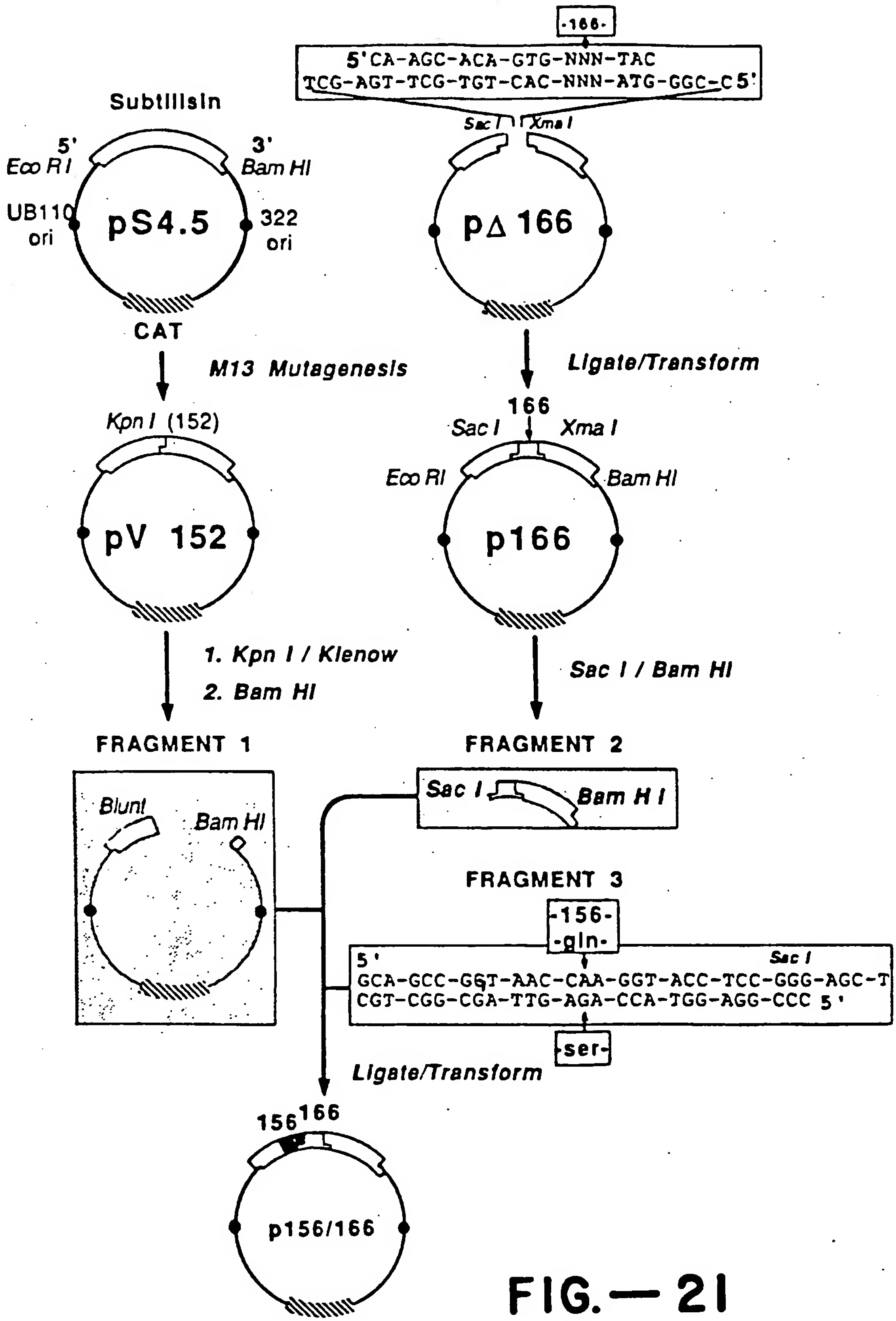

-166-
5'CA-AGC-ACA-GTG-NNN-TAC
TCG-AGT-TCG-TGT-CAC-NNN-ATG-GGC-C 5'
Subtilisin
5'
Eco RI
3'
Bam HI
Sac I
Xma I
UB110
ori
pS4.5
322
ori
pΔ 166
CAT
M13 Mutagenesis
Ligate/Transform
Kpn I (152)
166
Sac I
Xma I
Eco RI
Bam HI
pV 152
p166
1. Kpn I / Klenow
2. Bam HI
Sac I / Bam HI
FRAGMENT 1
FRAGMENT 2
Blunt
Bam HI
Sac I
Bam H I
FRAGMENT 3
-156-
-gln-
5'
Sac I
GCA-GCC-GGT-AAC-CAA-GGT-ACC-TCC-GGG-AGC-T
CGT-CGG-CGA-TTG-AGA-CCA-TGG-AGG-CCC 5'
-ser-
Ligate/Transform
156
166
p156/166

FIG.— 21

```
1. Codon number:                     211                    215     217            220
2. Wild type amino acid sequence:    Gly-Asn-Lys-Tyr-Gly-Ala-Tyr-Asn-Gly-Thr-Ser-Met-Ala
3. Wild type DNA sequence:        5'-GGA-AAC-AAA-TAC-GGG-GCG-TAC-AAC-GGT-ACG-TCA-ATG-GCA
                                     CCT-TTG-TTT-ATG-CCC-CGC-ATG-TTG-CCA-TGC-AGT-TAC-CGT-5'

                                                     *   *             *  **
4. pΔ217                          5'-GGA-AAC-AAA-TAC-GGC-GCC-TAC------GG-ATA-TCA-ATG-GCA
                                     CCT-TTG-TTT-ATG-CCG-CGG-ATG------CC-TAT-AGT-TAC-CGT-5'
                                                       Nar I               Eco RV

                                                      *                    *
5. pΔ217 cut with Nar I           5'-GGA-AAC-AAA-TAC-GG                   pA-TCA-ATG-GCA
   and Eco RI                        CCT-TTG-TTT-ATG-CCG-Gp                T-AGT-TAC-CGT-5'

                                                       *   ***             *
6. Cut pΔ217 ligated with         5'-GGA-AAC-AAA-TAC-GGC-GCG-NNN-AAC-GGT-ACA-TCA-ATG-GCA
   cassettes:                        CCT-TTG-TTT-ATG-CCG-CGC-NNN-TTG-CCA-TGT-AGT-TAC-CGT-5'

                                                    *   *     *  **
7. Mutagenesis primer             5'-GA-AAC-AAA-TAC-GGC-GCC-TAC-GGA-TAT-CAA-TGG-CAT-3'
   for pΔ217:

8. Mutants made:                  All 19 at 217
```

FIG.—22

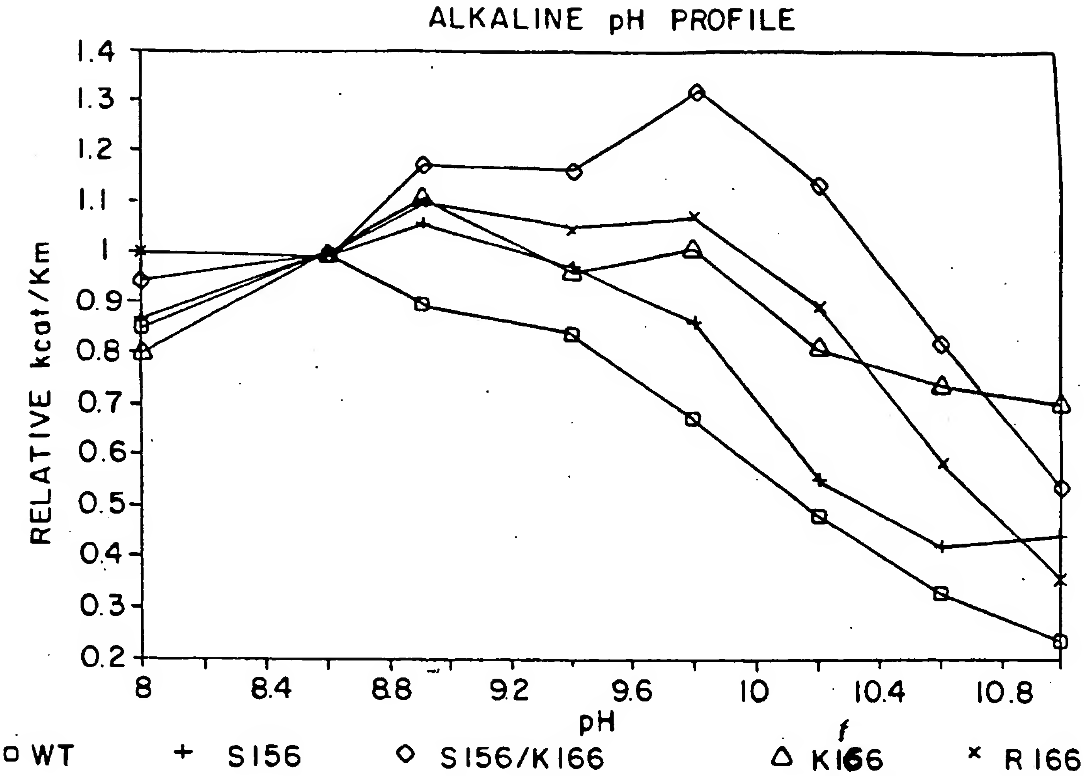


FIG. — 23A

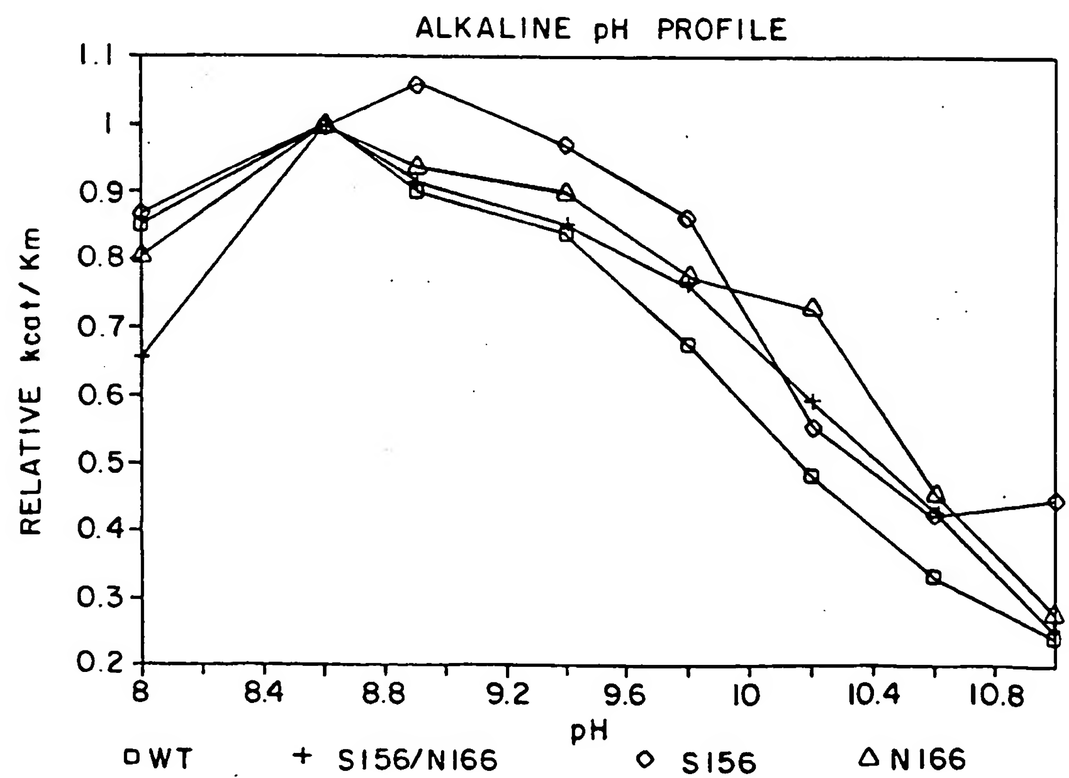


FIG. — 23B

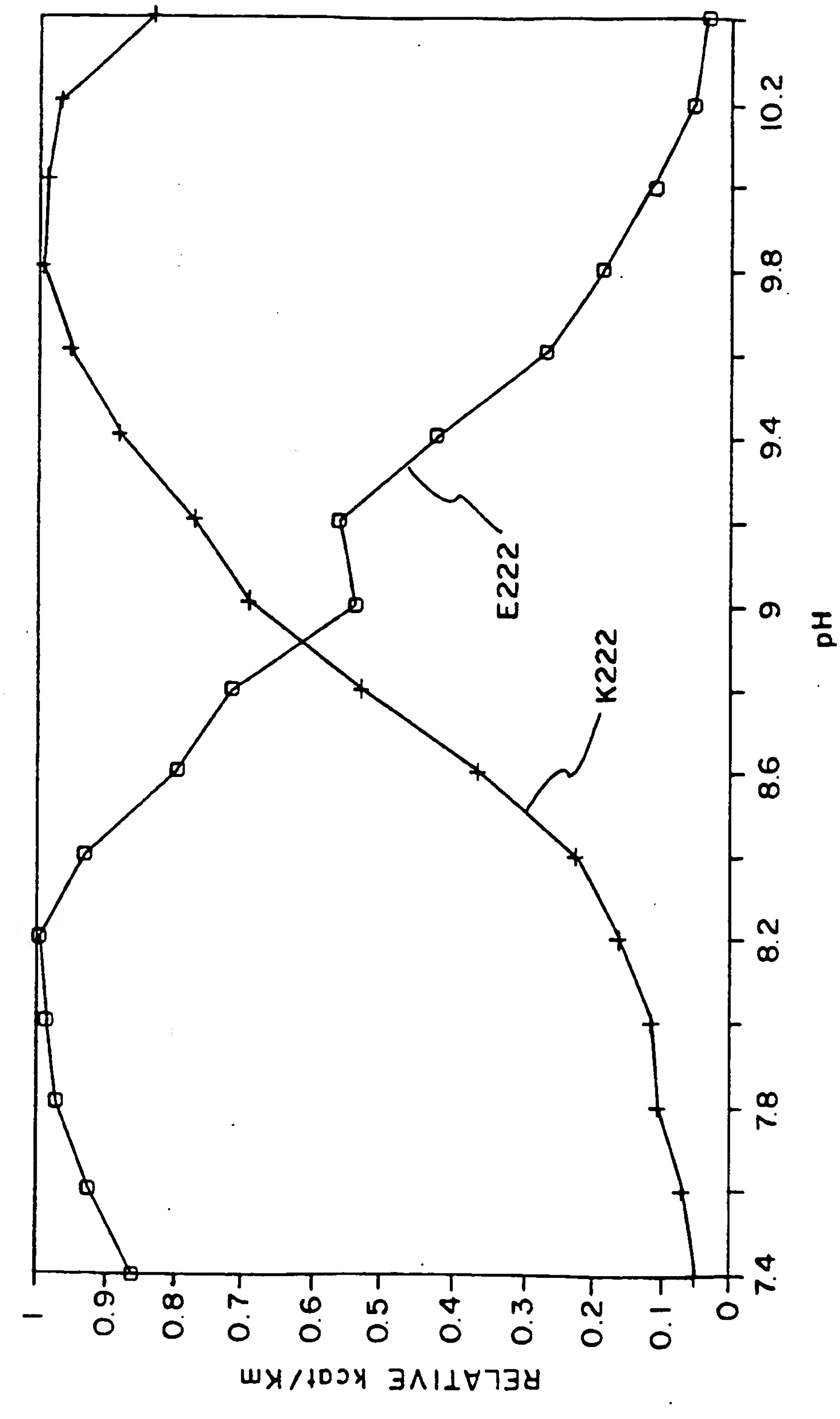
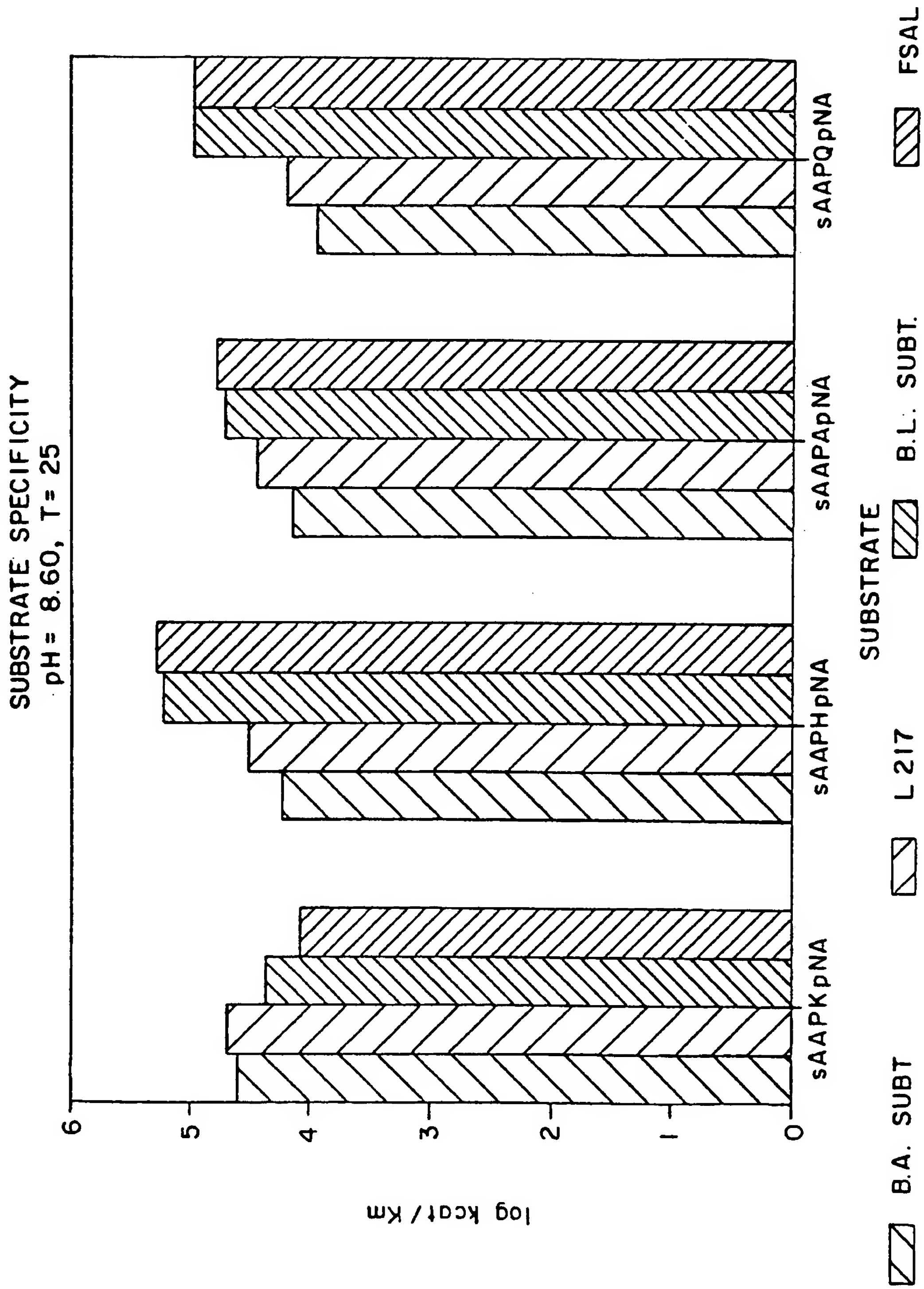

RELATIVE kcat/Km
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
7.4
7.8
8.2
8.6
9
9.4
9.8
10.2
pH
K222
E222

FIG. — 24

```
1. Codon number:                     91              95                  100
2. Wild type amino acid sequence:    Tyr-Ala-Val-Lys-Val-Leu-Gly-Ala-Asp-Gly-Ser
3. Wild type DNA sequence:         5'-TAC-GCT-GTA-AAA-GTT-CTC-GGT-GCT-GAC-GGT-TCC
                                      ATG-CGA-CAT-TTT-CAA-GAG-CCA-CGA-CTG-CCA-AGG-5'

                                           *  *                   *    *
4. pΔ95:                           5'-TAC-GCG-T-----------CTC-GCT-GCA-GAC-GGT-TCC
                                      ATG-CGC-A-----------GAG-CGA-CGT-CTG-CCA-AGG-5'
                                         Mlu I                       Pst I

                                          *                          *
5. pΔ95 cut with Mlu I and Pst I   5'-TA                              pGAC-GGT-TCC
                                      ATG-CGCp                      A-CGT-CTG-CCA-AGG-5'

                                          *                          *
6. Cut pΔ95 ligated with cassettes: 5'-TAC-GCG-GTA-AAA-GTT-CTC-GGT-GCA-GAC-GGT-TCC
                                      ATG-CGC-CAT-TTT-CAA-GAG-CCA-CGT-CTG-CCA-AGG-5'

                                                     *  *     *    *
7. Mutagenesis primer for pΔ95:    5'-CA-TCA-CTT-TAC-GCG-T-CTC-GCT-GCA-GAC-GGT-TCC

8. Mutants made:                   C94, C95, D96
```

FIG.—25



SUBSTRATE SPECIFICITY
pH = 8.60, T = 25
log kcat / Km
6
5
4
3
2
1
0
sAAPKpNA
sAAPHpNA
sAAPApNA
sAAPQpNA
SUBSTRATE
B.A. SUBT
L 217
B.L. SUBT.
FSAL

FIG.—26

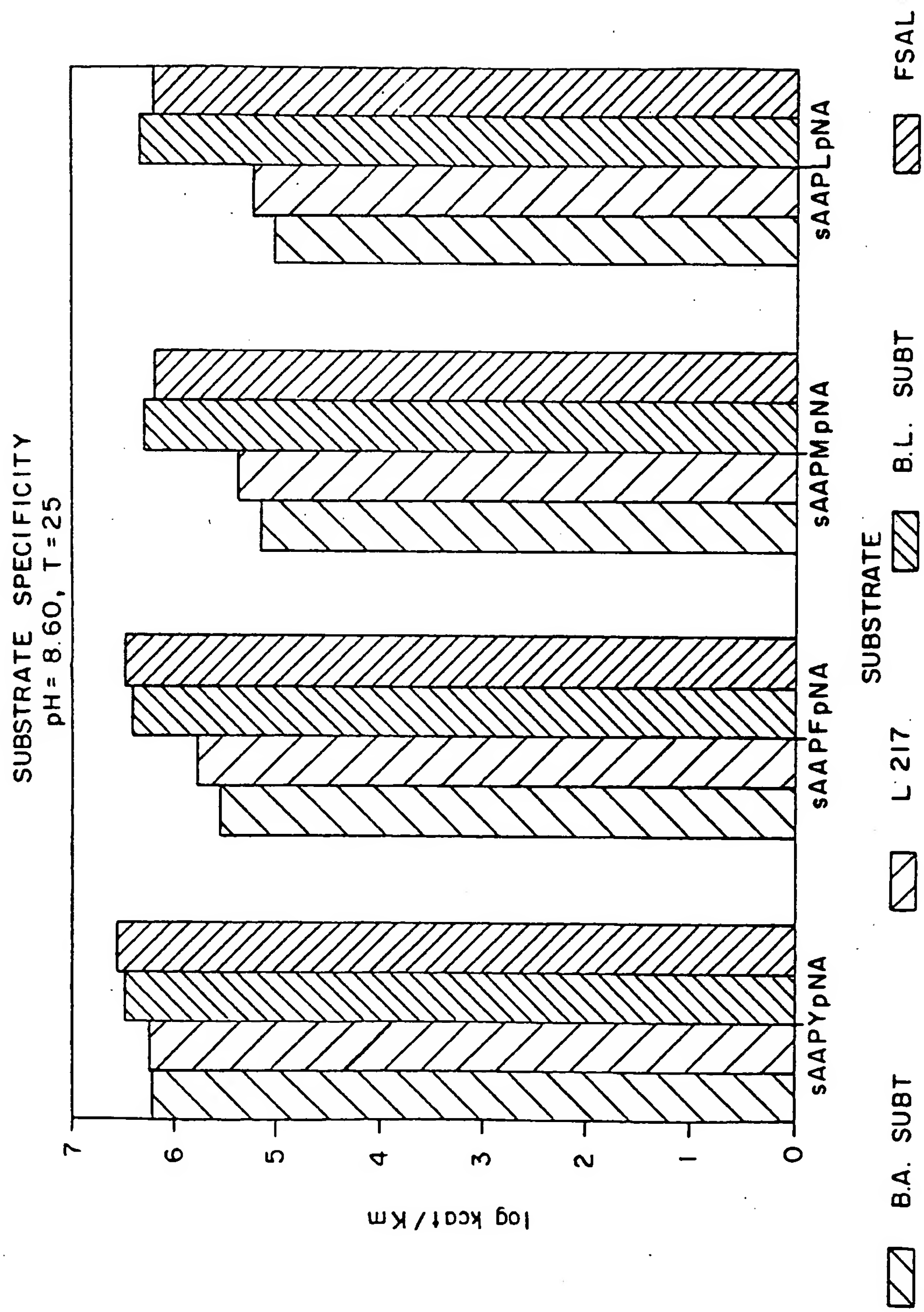

SUBSTRATE SPECIFICITY
pH= 8.60, T=25
log kcat/Km
0
1
2
3
4
5
6
7
sAAPYpNA
sAAPFpNA
sAAPMpNA
sAAPLpNA
SUBSTRATE
B.A. SUBT
L 217
B.L. SUBT
FSAL

FIG.—27

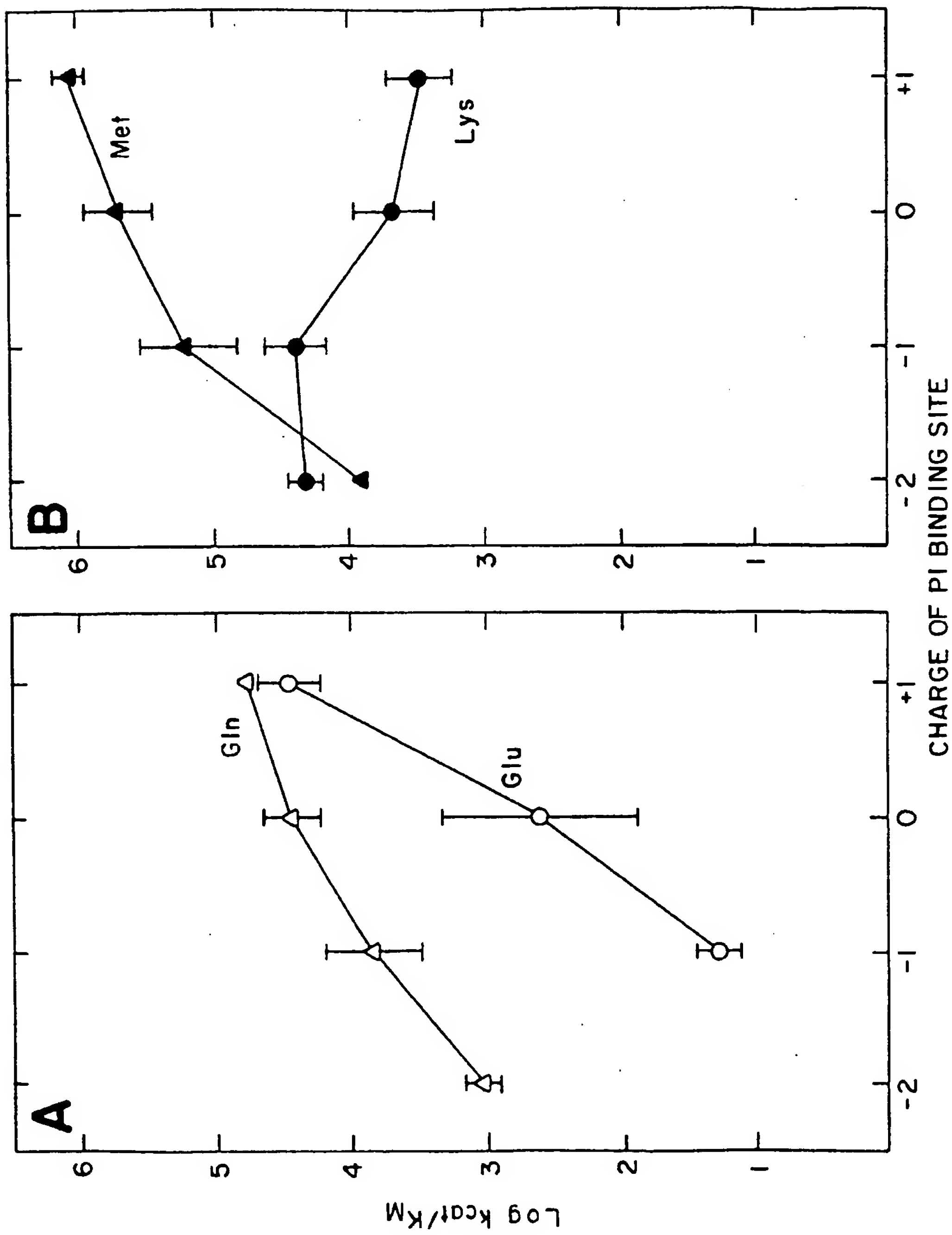

A
B
Gln
Glu
Met
Lys
Log kcat/KM
6
5
4
3
2
1
-2
-1
0
+1
CHARGE OF PI BINDING SITE

FIG. —28

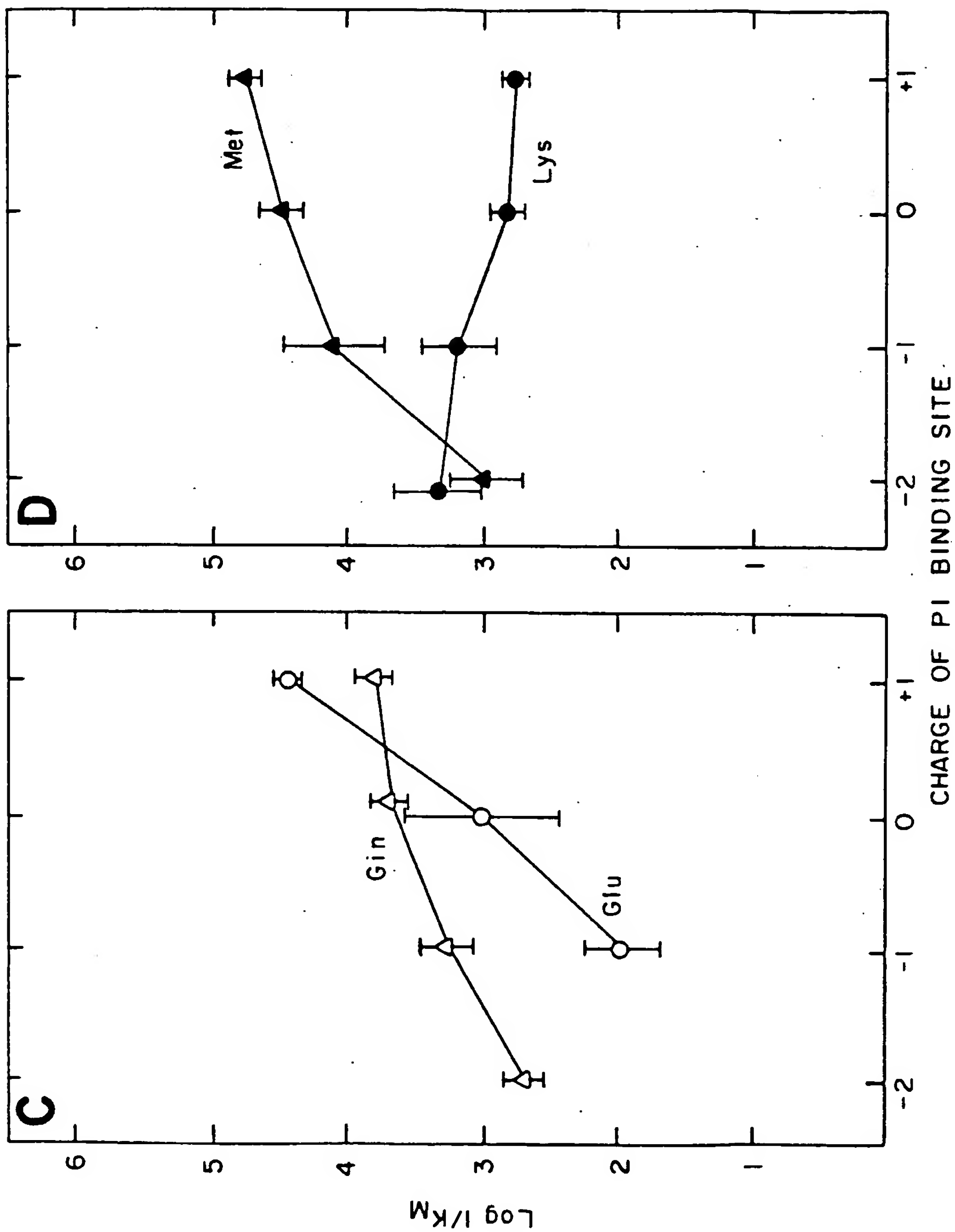

C
D
Gln
Glu
Met
Lys
Log 1/K$_M$
CHARGE OF PI BINDING SITE
6
5
4
3
2
1
-2
-1
0
+1

FIG. -28

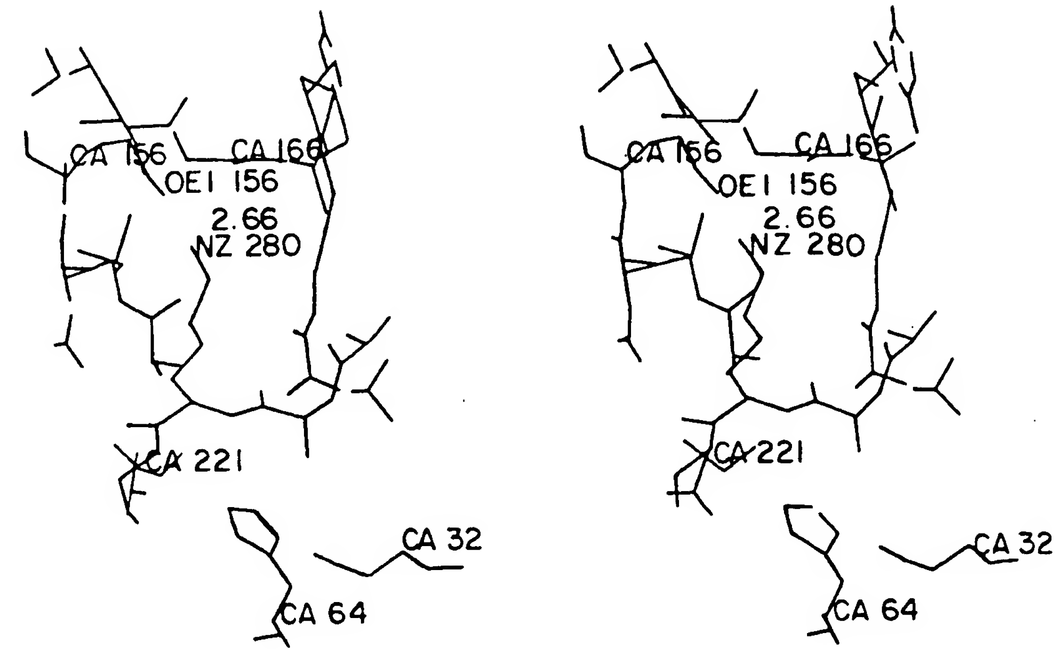


FIG. — 29A

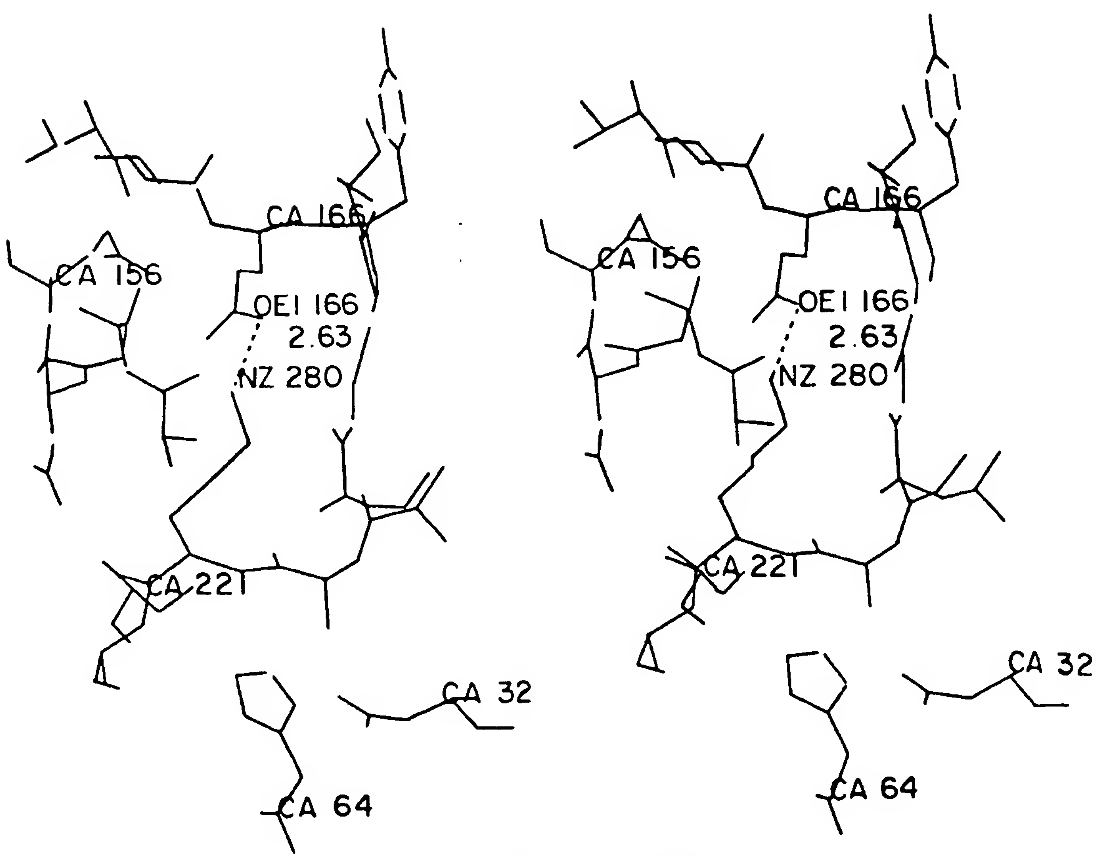


FIG. — 29B

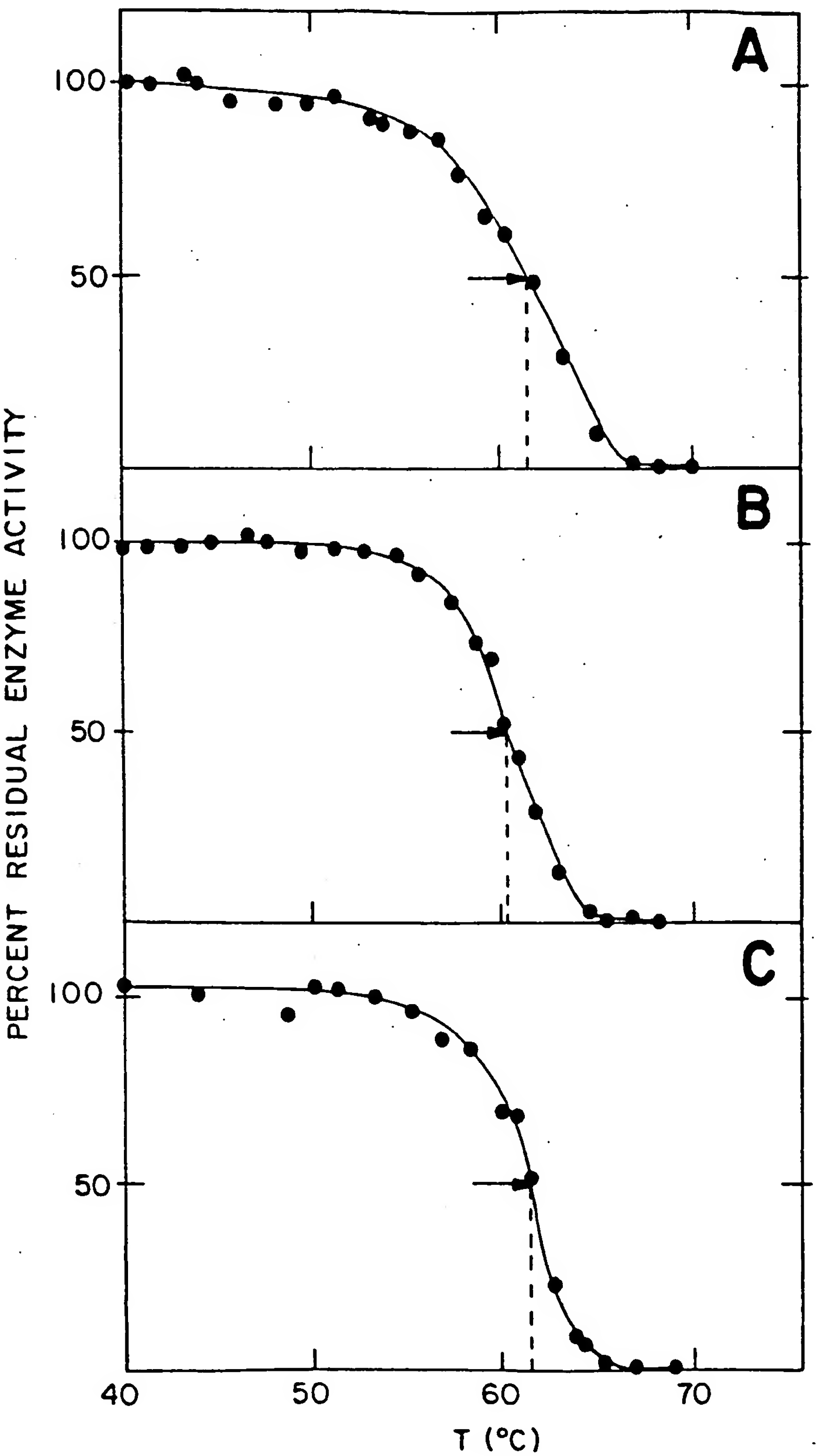

A
B
C
100
50
100
50
100
50
PERCENT RESIDUAL ENZYME ACTIVITY
40
50
60
70
T (°C)

FIG.—30

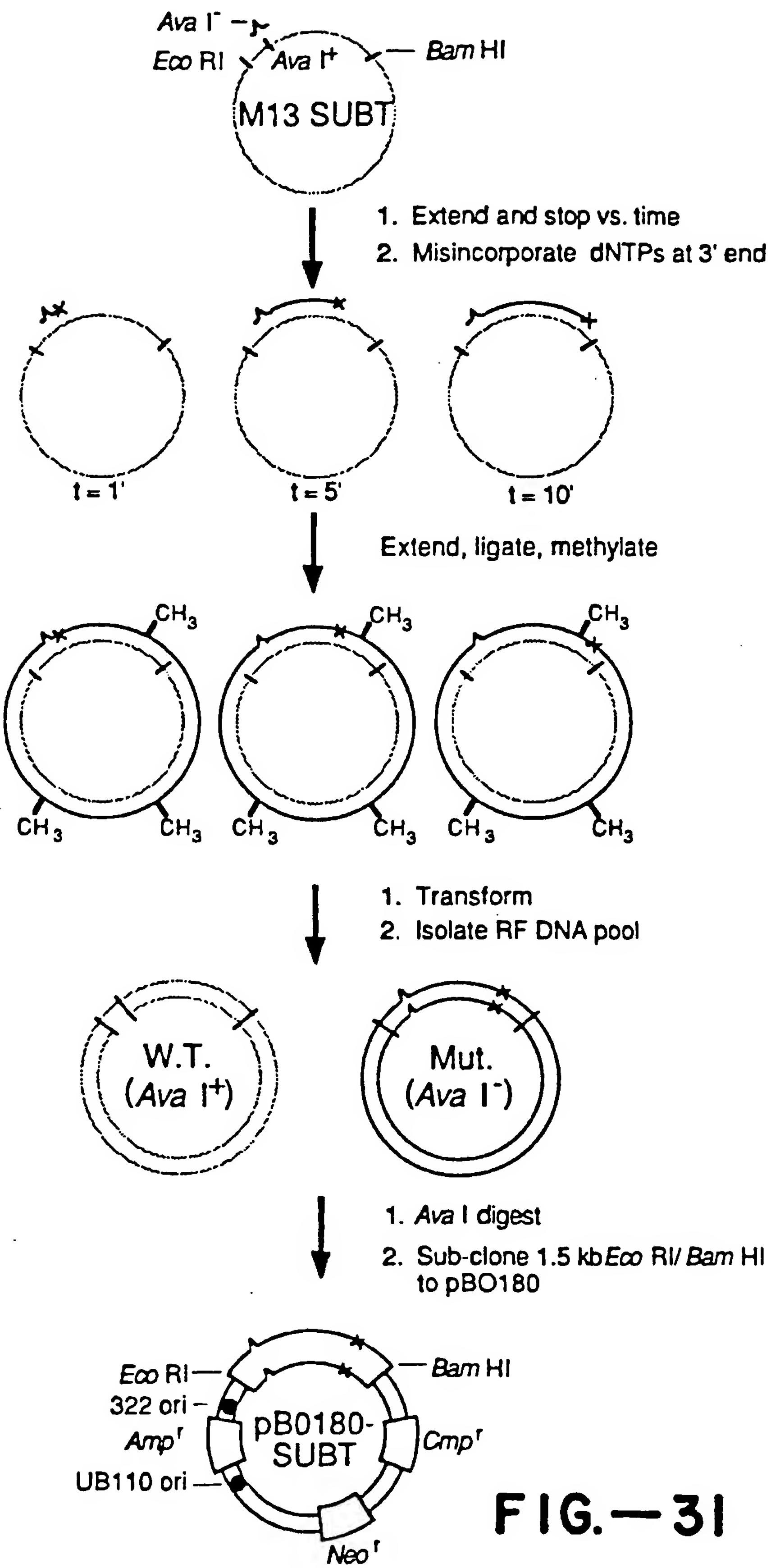
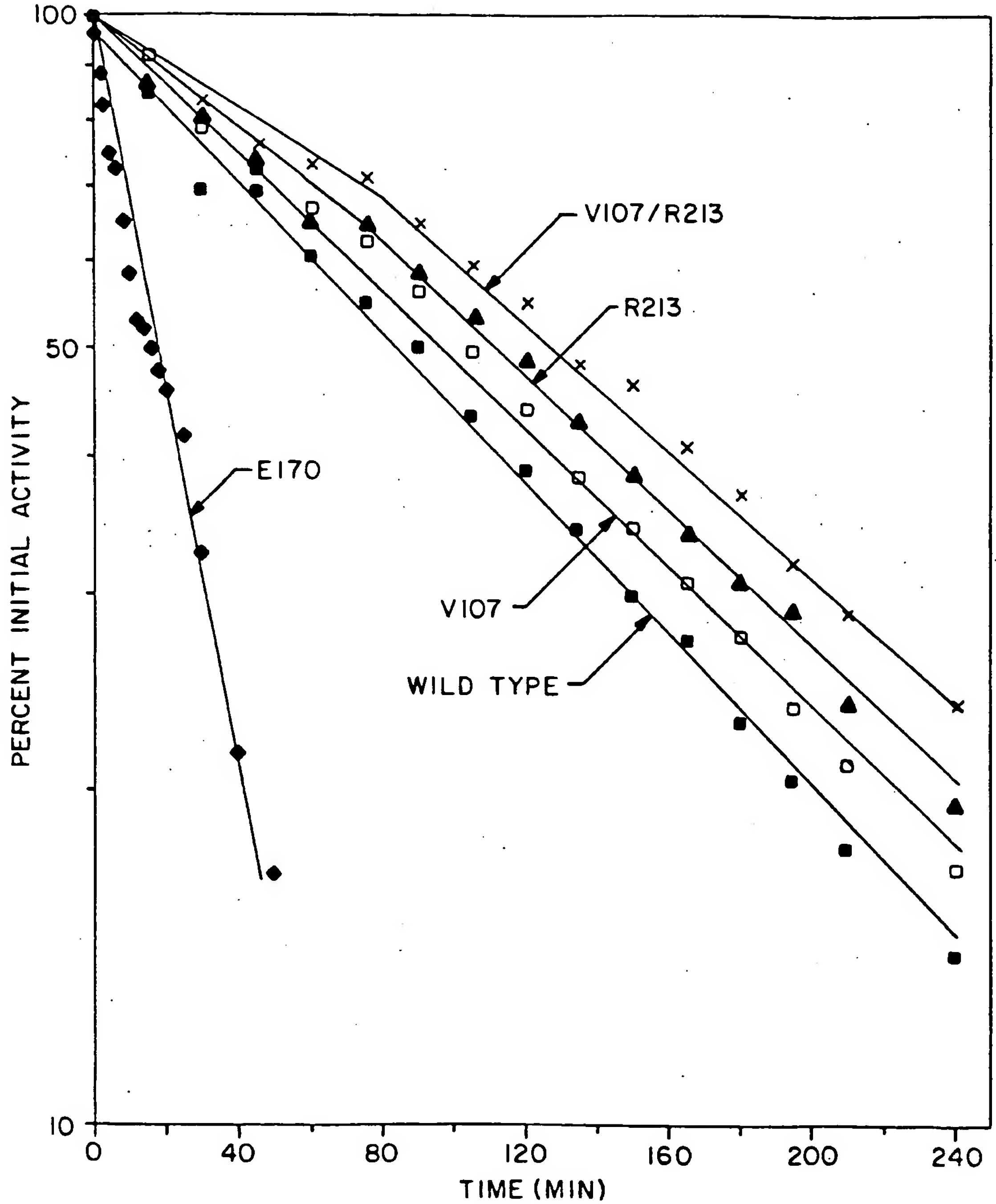

Ava I⁻
Eco RI
Ava I⁺
Bam HI
M13 SUBT
1. Extend and stop vs. time
2. Misincorporate dNTPs at 3' end
t = 1'
t = 5'
t = 10'
Extend, ligate, methylate
CH3
1. Transform
2. Isolate RF DNA pool
W.T. (Ava I⁺)
Mut. (Ava I⁻)
1. Ava I digest
2. Sub-clone 1.5 kb Eco RI/ Bam HI to pBO180
Eco RI
322 ori
Amp^r
UB110 ori
pB0180-SUBT
Bam HI
Cmp^r
Neo^r

FIG.—31



100
50
10
PERCENT INITIAL ACTIVITY
0
40
80
120
160
200
240
TIME (MIN)
V107/R213
R213
E170
V107
WILD TYPE

FIG. - 32

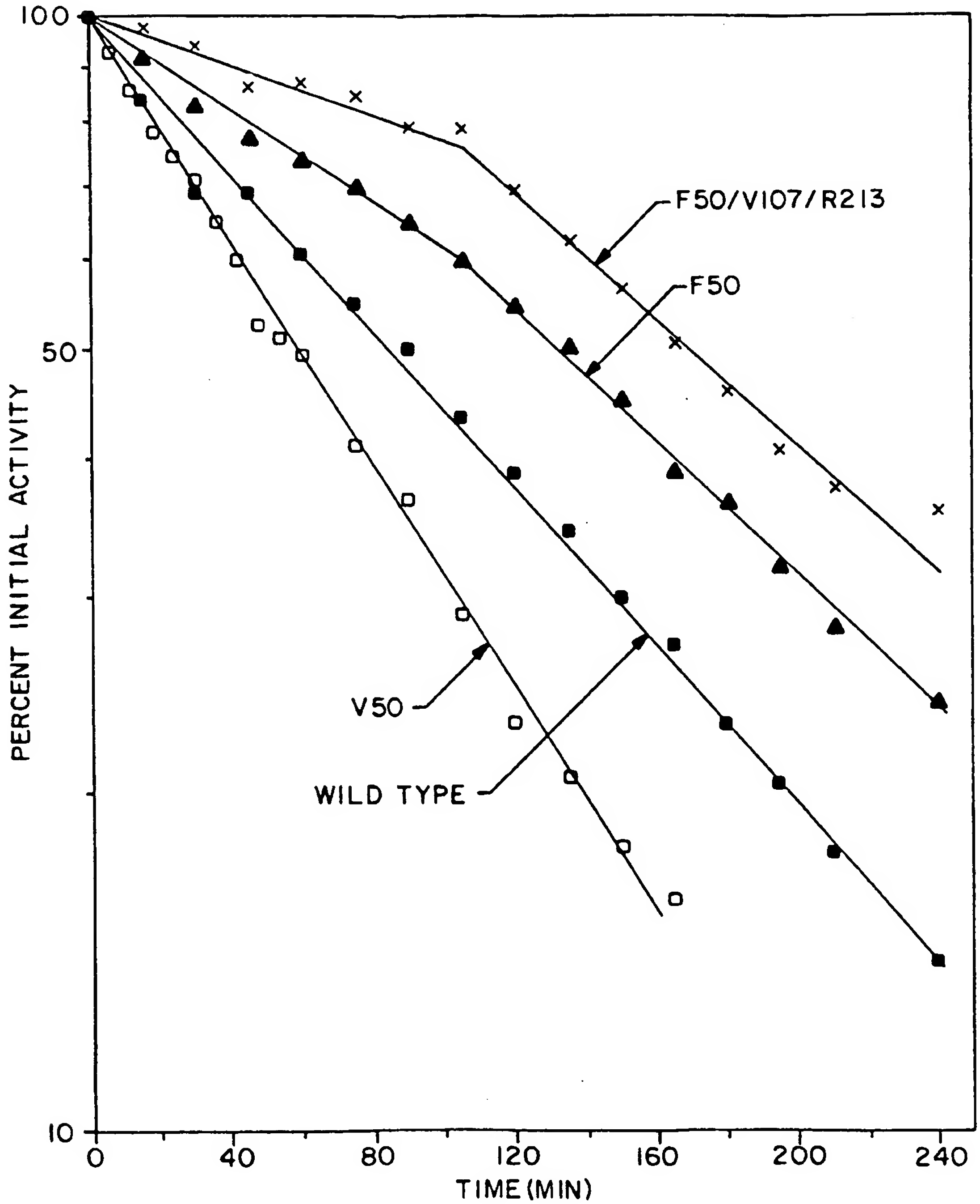

100
50
10
PERCENT INITIAL ACTIVITY
0
40
80
120
160
200
240
TIME (MIN)
F50/V107/R213
F50
V50
WILD TYPE

FIG.-33

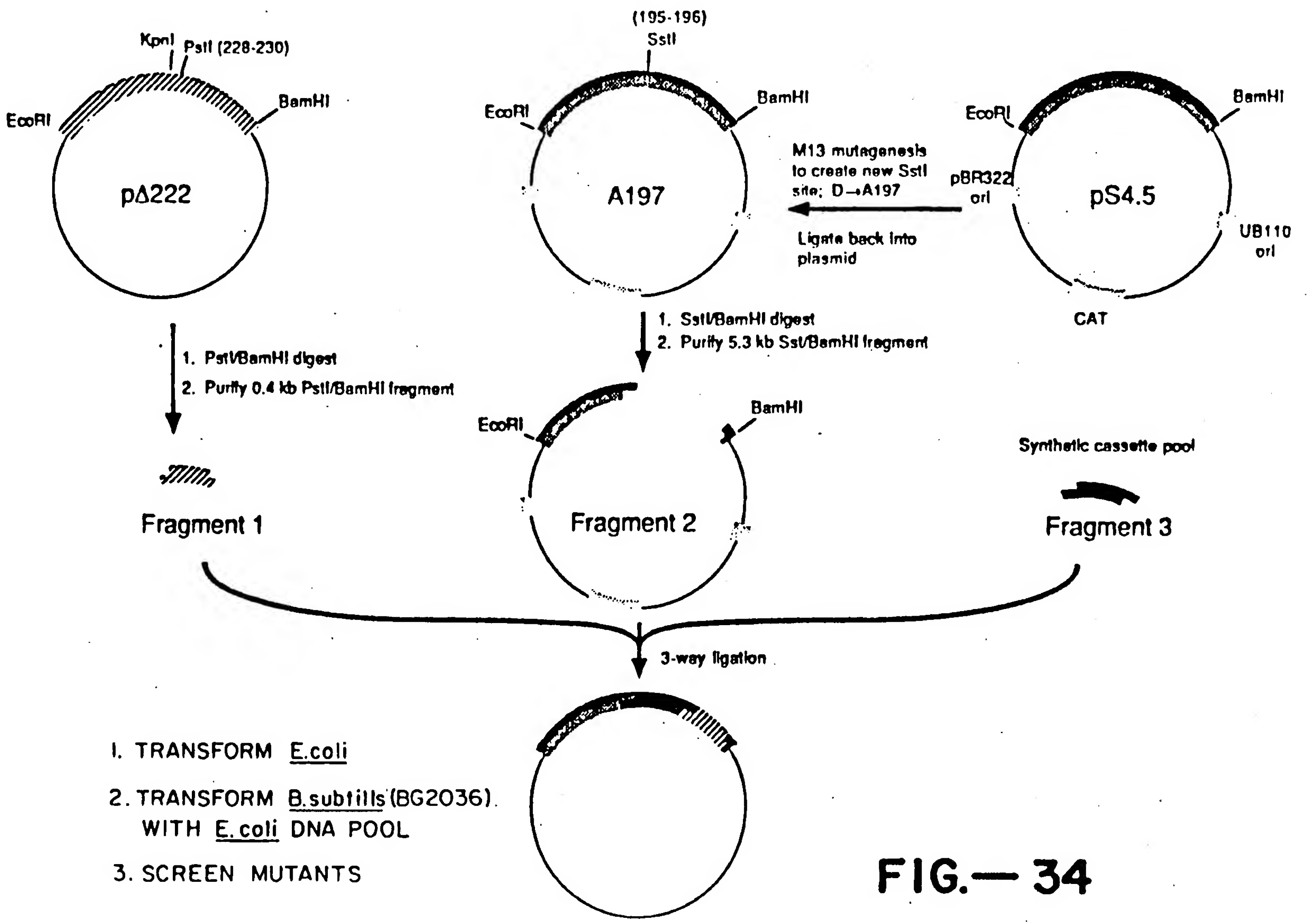

(195-196)
SstI
Kpnl
PstI (228-230)
EcoRI
BamHI
pΔ222
EcoRI
BamHI
A197
EcoRI
BamHI
pS4.5
pBR322 ori
UB110 ori
CAT
M13 mutagenesis to create new SstI site; D→A197
Ligate back into plasmid
1. PstI/BamHI digest
2. Purify 0.4 kb PstI/BamHI fragment
1. SstI/BamHI digest
2. Purify 5.3 kb SstI/BamHI fragment
EcoRI
BamHI
Synthetic cassette pool
Fragment 1
Fragment 2
Fragment 3
3-way ligation
1. TRANSFORM E.coli
2. TRANSFORM B.subtilis (BG2036) WITH E.coli DNA POOL
3. SCREEN MUTANTS

FIG.— 34

	195 ... 200 ... 206
W.T A.A.:	Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln
W.T. DNA:	GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA CTC GAA CTA CAG TAC CGT GGA CCG CAT AGA TAG GTT
pΔ222DNA:	GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA CTC GAA CTA CAG TAC CGT GGA CCG CAT AGA TAG GTT
A197 DNA:	GAG CTC GCA GTC ATG GCA CCT GGC GTA TCT ATC CAA CTC GAG CGT CAG TAC CGT GGA CCG CAT AGA TAG GTT *SstI*
Fragments from pΔ222 and A197 cut w/ *PstI*, *SstI*:	GAG-CT Cp
pΔ222, A197 cut & ligated w/ oligodeoxy-nucleotide pools:	GAG CTC GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA CTC GAG CTA CAG TAC CGT GGA CCG CAT AGA TAG GTT *SstI*

	207 ... 210 ... 218
W.T A.A.:	Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn
W.T. DNA:	AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC TCG TGC GAA GGA CCT TTG TTT ATG CCC CGC ATG TTG
pΔ222DNA:	AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC TCG TGC GAA GGA CCT TTG TTT ATG CCC CGC ATG TTG
A197 DNA:	AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC TCG TGC GAA GGA CCT TTG TTT ATG CCC CGC ATG TTG
Fragments from pΔ222 and A197 cut w/ *PstI*, *SstI*:	AGC ACG CTT CCC GGG AAC AAA TAC GGG GCG TAC AAC TCG TGC GAA GGG CCC TTG TTT ATG CCC CGC ATG TTG *SmaI*

	219 220 ... 230
W.T A.A.:	Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Ala
W.T. DNA:	GGT ACG TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG-3' CCA TGC AGT TAC CGT AGA GGC GTG CAA CGG CCT CGC-5'
pΔ222DNA:	GGT ACC TCA--------------CG CAC GCT GCA GGA GCG-3' CCA TGG AGT--------------GC GTG CGA CGT CCT CGC-5' *KpnI* *PstI*
A197 DNA:	GGT ACG TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG-3' CCA TGG AGT TAC CGT AGA GGC GTG CAA GTG CCT CGC-5'
Fragments from pΔ222 and A197 cut w/ *PstI*, *SstI*:	pGGA GCG-3' A CGT CCT CGC-5'
pΔ222, A197 cut & ligated w/ oligodeoxy-nucleotide pools:	GGT ACC TCA ATG GCA TCT CCG CAC GTT GCA GGA GCG-3' CCA TGG AGT TAC CGT AGA GGC GTG CAA CGT CCT CGC-5' *KpnI* *PstI* destroyed

Oligodeoxynucleotide pools synthesized with 2% contaminating nucleotides in each cycle to give ~15% of pool with 0 mutations, ~28% of pool with single mutations, and ~57% of pool with 2 or more mutations, according to the general formula $f = \frac{\mu^n}{n!} e^{-\mu}$.

FIG.—35

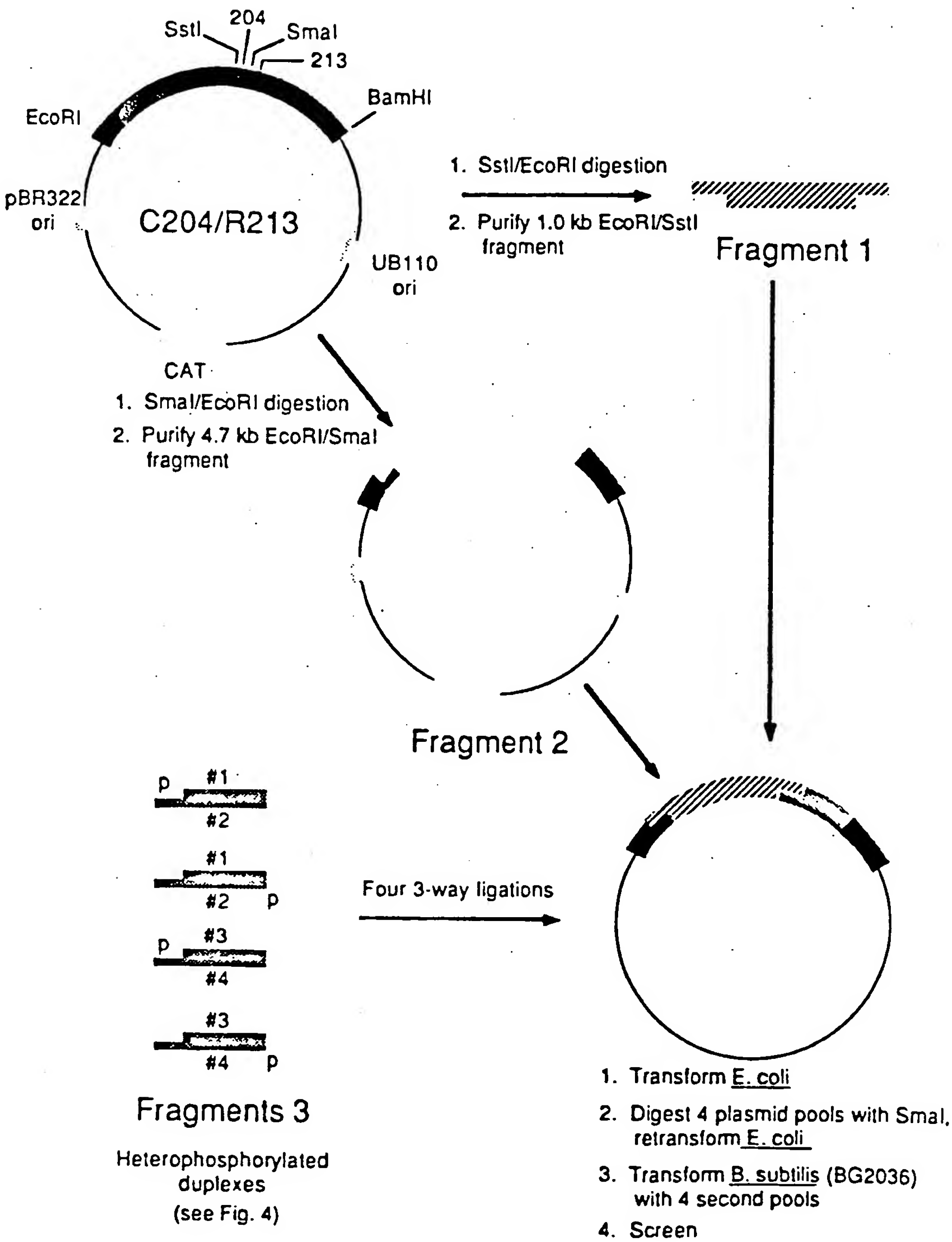

SstI
204
SmaI
213
BamHI
EcoRI
pBR322 ori
C204/R213
UB110 ori
CAT
1. SstI/EcoRI digestion
2. Purify 1.0 kb EcoRI/SstI fragment
Fragment 1
1. SmaI/EcoRI digestion
2. Purify 4.7 kb EcoRI/SmaI fragment
Fragment 2
p #1 #2
#1 #2 p
p #3 #4
#3 #4 p
Four 3-way ligations
Fragments 3
Heterophosphorylated duplexes
(see Fig. 4)
1. Transform E. coli
2. Digest 4 plasmid pools with SmaI, retransform E. coli
3. Transform B. subtilis (BG2036) with 4 second pools
4. Screen

FIG.—36

```
                        195                      200                 204                                     210           213
Wild type A.A.:         Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Glu Ser Thr Leu Pro Gly Asn Lys

Wild type DNA:       5'-GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA AGC ACG CTT CCT GGA AAC AAA-3'
                     3'-CTC GAA CTA CAG TAC CGT GGA CCG CAT AGA TAG GTT TCG TGC GAA GGA CCT TTG TTT-5'

C204/R213 DNA:       5'-GAG CTC GAT GTC ATG GCA CCT GGC GTA TGT ATC CAA AGC ACG CTT CCC GGG AAC AGA-3'
                     3'-CTC GAG CTA CAG TAC CGT GGA CCG CAT ACA TAG GTT TCG TGC GAA GGG CCC TTG TCT-5'
                            SstI                                                         SmaI

C204/R213 cut                                                                           GGG AAC AGA-3'
with SstI and SmaI:  5'-GAG CT                                                          CCC TTG TCT-5'
                     3'-C

C204/R213 cut and    5'-GAG CTC GAT CTC ATG GCA CCT GGG GTA     ATC CAG TCG ACG CTT CCT GGG AAC AGA-3'
ligated with oligo-  3'-CTC GAG CTA CAG TAC CGT GGA CCG CAT     TAG GTC AGC TGC GAA GGA CCC TTG TCT-5'
deoxynucleotide pools:      SstI                                        SalI        SmaI⁻

                                                 #1           #3
                             W,R,R, or G ←       NGG    or    NCC  →  S,P,T or A
           Stop,Y,H,Q,N,K,D or E ←  [G/C]TN     or   [G/C]AN  →  L,F,I,V or M
                                     #2               #4
```

FIG.—37